INVESTIGATING THE EFFECT OF PHYTOPLANKTON ON BACTERIAL COMMUNITY COMPOSITION ACROSS DIFFERENT ENVIRONMENTAL CONTEXTS

BY

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DISSERTATION

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ABSTRACT

The following dissertation encompasses a series of investigations aimed at testing the hypothesis that phytoplankton seasonal succession effects changes in bacterial community composition and characterizing interactions between phytoplankton and bacteria across different environmental contexts. Observational and experimental approaches were combined to determine the effect of phytoplankton on the composition of the bacterial community in three humic lakes in Northern Wisconsin where correlated patterns of community change between phytoplankton and bacteria have previously been observed. Because community-level analyses can aggregate ecologically distinct bacterial populations into a single operational taxonomic unit, the effect of phytoplankton on the composition of subtypes within the cosmopolitan freshwater bacterial genus *Polynucleobacter* was additionally determined. Changes in the abundance of phytoplankton populations explained a substantial amount of temporal variation in *Polynucleobacter* composition, similar to variation in total bacterial community composition explained by phytoplankton. Full factorial algal exchange experiments combining bacteria from each lake with phytoplankton from one of the lakes or a no-phytoplankton control confirmed the effect of phytoplankton presence and composition on the composition of the bacterial community and *Polynucleobacter* subtypes. Bacterial community response appeared to be primarily due to phytoplankton enriching for specific free-living bacteria. However, phytoplankton-associated bacteria and *Polynucleobacter* subtypes did contribute to observed differences between phytoplankton treatments and corresponding no-phytoplankton controls.

Light and temperature vary temporally and with depth in lakes and have the potential to modify interactions with phytoplankton as well as influence bacteria directly. To determine how phytoplankton, light, and temperature combine to affect bacterial communities, a multi-factorial

experiment was conducted. Bacteria from two of the humic lakes were combined with phytoplankton assemblages from each lake ("home" or "away") or a no-phytoplankton control and incubated for 5 days under all combinations of light (surface, ~25% surface irradiance) and temperature (5 levels from 10°C to 25°C). Light had a direct effect on bacterial community composition, potentially due to stimulating the growth of phototrophic bacteria. Temperature effects were largely phytoplankton-mediated and the effect of temperature was greatest for "away" phytoplankton treatments. We hypothesize that the enhanced effect of "away" phytoplankton on bacteria is due to the availability of a different pool of exudates than bacteria had become acclimated to in their "home" lake.

To determine support for phytoplankton exudates to affect bacterial succession, the *glcD* gene, which indicates genetic potential for bacteria to use the algal exudate glycolate, was characterized in Emerald Lake, an oligotrophic high-elevation lake that shifts from primarily terrestrial-derived organic matter following ice-off to primarily phytoplankton-derived organic matter. *glcD* genes were not detected in early season samples when phytoplankton-derived resources were scarce. Following this period, *glcD* gene composition exhibited significant changes through time, providing support for exudate-mediated phytoplankton effects and strengthening the evidence for shifts in dissolved organic matter to structure bacterial communities. Results from these studies contribute to the ability to predict changes in bacterial community composition and determine underlying processes, thereby providing a backdrop for examining mechanisms that create and maintain diversity and enhancing the ability to forecast community responses to environmental change.

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CHAPTER 1

INTRODUCTION

BACKGROUND

Importance of understanding microorganisms in a community context

Microbial processes (e.g., respiration, production, nitrogen fixation, methanogenesis) regulate energy flow and the cycling of carbon and nutrients in ecosystems (Falkowski et al., 2008; Bodelier, 2011). While much has been and continues to be learned by taking a "black box" approach that studies these processes without considering microbial community composition, the relationship between predictor variables and process rates is not necessarily straightforward. For example, in an experiment examining arctic lake bacterial production as a function of temperature, Adams et al. (2010) observed two temperature optima, not the general increase with temperature that would be expected if production were independent of community composition. Over the experimental incubation, mesocosms incubated at each temperature developed a distinct bacterial community composition (Adams et al., 2010). Effects of bacterial community composition on production have been observed in other systems as well (Judd et al., 2006; Bertilsson et al., 2007). The distribution and abundance of organisms in the environment, including those carrying out desired functions, is dynamic and affected by interactions among species in addition to environmental factors and stochastic processes (Morin, 1999). Thus, a framework for predicting microbial community patterns and dynamics has potential application for predicting how microbial processes and ecosystem function will change over time.

Planktonic seasonal succession in lake ecosystems

Temperate lakes are ideal systems for studying factors that affect community composition. Physical, chemical, and biotic changes occur seasonally due to lake stratification and mixing. These changes result in annually repeated patterns of community assembly or seasonal succession of planktonic communities (Sommer et al., 2012). Seasonal succession of phytoplankton and zooplankton has been observed over decades, and mechanisms for generally observed patterns have been described in the plankton ecology group (PEG) model published by Sommer and colleagues (1986) and revisited by Sommer and colleagues (2012). Investigations of community patterns in the context of phytoplankton functional traits (e.g., maximum growth rate, light and nutrient acquisition and use, and temperature sensitivity) show promise for conferring the ability to predict phytoplankton community structure (Litchman and Klausmeier, 2008; Edwards et al., 2013).

Similar to phytoplankton seasonal succession, seasonally repeating patterns of bacterial community composition have been observed in multi-year observations of eutrophic Lake Mendota (Shade et al., 2007; Kara et al., 2013) and oligotrophic Emerald Lake (Nelson, 2009). Remarkably consistent patterns of community assembly have also been observed in a humic lake in Taiwan following multiple typhoon-induced mixing events, which reset the bacterial community with some similarity to the reset caused by spring mixing (Jones et al., 2008; Shade et al., 2010). A number of factors have been proposed as affecting temporal patterns in bacterial community composition including: temperature (Shade et al., 2007; Adams et al., 2010), organic matter source (autochthonous vs. terrestrial) (Crump et al., 2003; Nelson, 2009), grazing pressure (Pernthaler, 2005; Kent et al., 2006), and phytoplankton composition (Kent et al., 2004; Kent et al., 2007; Šimek et al., 2008; Paver and Kent, 2010). Kent and colleagues (2007) tested the

ability of meteorological (air temperature, photosynthetically active radiation, and precipitation), environmental (water temperature, dissolved oxygen, total nitrogen and total phosphorus, dissolved organic carbon, and pH), and abundance of phytoplankton populations to explain temporal patterns of change in bacterial communities in six humic lakes. Changes in the composition of the bacterial community were significantly correlated with changes in phytoplankton community composition (Kent et al., 2007). Additionally, the abundance of phytoplankton populations and covariation between phytoplankton populations and environmental factors could explain over half of the variation in bacterial communities (Kent et al., 2007). These correlated patterns of change led to the hypothesis that phytoplankton seasonal succession induces changes in bacterial community composition in lakes.

Mechanisms of phytoplankton influence on lake bacterial community composition

Interactions between phytoplankton and bacteria range from antagonistic to competitive to mutualistic (Cole, 1982). Phytoplankton can affect bacterial communities by providing a source of organic matter, serving as a habitat (or host) to epiphytic or endophytic bacteria, grazing bacteria (in the case of mixotrophic phytoplankton), releasing inhibitory substances (e.g., antibiotics), and altering the pH of the microenvironment (Fig. 1.1)(Cole, 1982). In turn, bacteria can affect phytoplankton growth through remineralizing nutrients, releasing stimulatory products (e.g. vitamins), decreasing dissolved oxygen availability, and causing algal cells to lyse (Cole, 1982). The following phytoplankton interactions have been reviewed further due to their documented potential to affect bacterial community composition: providing a source of organic matter, grazing by mixotrophs, and serving as a habitat.

Phytoplankton-derived organic matter can become available to biota through exudate release from living cells (Baines and Pace, 1991), grazer 'sloppy feeding' (Jumars et al., 1989), auto or viral lysis (Bratbak et al., 1994; Brussaard et al., 2005), and following cell senescence and death (van Hannen et al., 1999). Phytoplankton-derived organic matter is generally species-specific in composition due to differences in physiology and biochemistry [e.g., storage products (Meeuse, 1962), and cell wall composition (Kreger, 1962)]. Phytoplankton-derived organic matter is a temporally dynamic resource due to phytoplankton seasonal succession (Sommer et al., 1986; Anneville et al., 2002; Graham et al., 2004). Changes in the expression and composition of carbohydrate-active enzymes over the course of marine phytoplankton blooms supports the model of substrate-mediated phytoplankton influence (Arrieta and Herndl, 2002; Teeling et al., 2012).

Exudates make up a substantial proportion of photo-assimilated carbon in phytoplankton cultures (Hellebust, 1965) and natural phytoplankton communities (Baines and Pace, 1991). In lakes, extracellular release of algal exudates averages between 5 and 41% of primary productivity (see Baines and Pace, 1991 for review). Exudate quantity and composition is affected by phytoplankton species (Hellebust, 1965; Fogg, 1983; Myklestad, 1995), nutrient availability (Hama and Honjo, 1987; Parker and Armbrust, 2005; Urbani et al., 2005), light availability (Morris and Skea, 1978; Parker and Armbrust, 2005; Panzenbock, 2007), and temperature (Parker and Armbrust, 2005). It is estimated that bacteria utilize algal exudates within hours of release (Maurin et al., 1997; Sadro et al., 2011). Changes in bacterial community composition have been observed following incubation with exudates from different phytoplankton species (Sarmento and Gasol, 2012) and phytoplankton grown under different phosphorus conditions (Puddu et al., 2003).

Characterizing bacteria that utilize algal exudates in the environment can be difficult because many phytoplankton-derived substrates in a lake (e.g., glucose) can have multiple origins other than phytoplankton. One exception is the commonly released algal exudate glycolate, which is a product of photorespiration (Hellebust, 1965; Fogg, 1983; Oliver, 1998). Due to the rapid utilization of glycolate (Wright, 1975), it is unlikely that glycolate available in pelagic habitats originated from outside the lake. Thus, glycolate-utilizing bacteria are a subset of the total bacterial community that can be specifically linked to algal exudates and autochthonous resources. Bacteria with genetic potential to use glycolate (putative glycolate-utilizing bacteria) can be characterized using the *glcD* gene, which encodes the D subunit of the enzyme glycolate oxidase (Lau and Armbrust, 2006). The composition of putative glycolate-utilizing bacteria has been observed to correlate with changes in phytoplankton community composition in lakes (Paver and Kent, 2010) and during a marine phytoplankton bloom (Lau et al., 2007).

Phytoplankton can additionally affect bacterial community composition by providing a habitat for bacteria. Phytoplankton-associated bacteria include epiphytic bacteria attached to cells and endophytic bacteria living within cells (Cole, 1982). Using electron and fluorescence microscopy, researchers have observed that bacteria are commonly associated with various algal taxa, including filamentous desmids and dinoflagellates (e.g., Fisher and Wilcox, 1996; Biegala et al., 2002). The microenvironment surrounding phytoplankton cells appears to select for specific bacteria. For example, bacterial populations on cultured phytoplankton cells were more similar among *Alexandrium* spp. cultures from different geographic regions than between *Alexandrium* spp. and other non-toxic phytoplankton in the Gulf of Maine (Jasti et al., 2005).

Through enhancing the proliferation of attached bacteria, phytoplankton can affect bacterial community composition.

Finally, mixotrophic phytoplankton may be able to affect bacterial community composition through selectively grazing bacteria. Mixotrophic phytoplankton combine energy acquisition strategies by carrying out photosynthesis (autotrophic) and consuming bacteria (heterotrophic). Mixotrophy is common across many different phytoplankton groups, including *Cryptomonas* spp. and *Dinobryon* spp. (Palsson and Graneli, 2003; Flynn et al., 2013). In an oligotrophic lake, differences in the rate of ingestion of bacteria by mixotrophic phytoplankton were observed diurnally (highest in the afternoon, lowest at night) and seasonally (highest in winter) (Palsson and Graneli, 2003). Experimentally, it has been observed that temperature (Wilken et al., 2013) and light availability (Caron et al., 1993; Jones et al., 1993; Hansen and Nielsen, 1997; Holen, 1999; Skovgaard et al., 2000) can affect consumption of bacteria by phytoplankton. Because bacteria differ in their susceptibility to grazing, enhanced grazing by mixotrophic phytoplankton has the potential to affect bacterial community composition (Pernthaler, 2005).

OVERVIEW

This dissertation is composed of a series of investigations that test the hypothesis that phytoplankton succession causes changes in bacterial community composition and characterizes interactions between phytoplankton and bacteria across different environmental contexts. To study the effects of phytoplankton on bacteria, I combined weekly environmental time series observations, experiments manipulating the presence and composition of phytoplankton, and a natural experiment of a shift from terrestrial-derived to phytoplankton-derived organic matter.

Chapters 2-4 investigate microbial communities in humic lakes in Vilas County, Wisconsin, where Kent and colleagues (2007) have previously described correlated patterns of community change between phytoplankton and bacteria. The study site for Chapter 5 is Emerald Lake, a high-elevation oligotrophic system with an annual shift from predominantly terrestrial-derived to predominantly phytoplankton-derived dissolved organic matter (Nelson, 2009).

In Chapter 2, I combined environmental time series observations and an algal exchange experiment to test the hypothesis that interactions between specific phytoplankton and bacterial populations affect bacterial community dynamics. The algal exchange experiment assessed change in the composition of bacterial communities (<1 μm size fraction) that originated from one of three lakes following incubation with native phytoplankton assemblages (20-100 μm size fraction) from each lake and a no-phytoplankton control in a full factorial design. Following incubation, bacterial community composition depended on whether they were incubated with phytoplankton and the source lake of the phytoplankton assemblage. By comparing specific bacterial operational taxonomic units (OTUs) with inferred responses to phytoplankton in the environment and the experiment, I provide evidence that interactions between phytoplankton and specific bacteria contribute to bacterial community succession. Additionally, I demonstrate that differences observed between phytoplankton treatments and corresponding no-phytoplankton treatments were primarily due to the response of free-living bacteria. *Environmental Microbiology* published Chapter 2 in September 2013.

Because community-level analyses can aggregate ecologically distinct bacterial populations into the same OTU (Jaspers and Overmann, 2004; Koeppel et al., 2008; Melendrez et al., 2011), in Chapter 3, I tested the hypothesis that phytoplankton affect the composition of subtypes within the bacterial genus *Polynucleobacter*. *Polynucleobacter* has a cosmopolitan

distribution in freshwater ecosystems (Jezberová et al., 2010; Newton et al., 2011) and is abundant in the humic lakes studied. *Polynucleobacter* subtype composition was characterized in time-series observations and a two-lake algal exchange experiment using DNA sequencing and fingerprinting of the cytochrome c oxidase gene amplified using *Polynucleobacter*-specific PCR primers. An average of 30% of the temporal variation observed in *Polynucleobacter* composition could be explained by changes in the abundance of phytoplankton populations. Phytoplankton treatment-dependent changes in *Polynucleobacter* composition experimentally confirmed that phytoplankton have a subtype-specific effect on organisms in the *Polynucleobacter* genus. Chapter 3 has been submitted to *Environmental Microbiology*.

In Chapter 4, I describe the results of a multi-factorial experiment testing the direct and interactive effects of phytoplankton, temperature, and light on bacterial community composition. Bacteria from two humic lakes were combined with phytoplankton assemblages from each lake ("home" or "away") or a no-phytoplankton control and incubated for 5 days under all combinations of light (surface, ~25% surface irradiance) and temperature (5 levels from 10°C to 25°C). Direct effects of light were observed, potentially due to stimulating the growth of phototrophic bacteria. Temperature effects were largely phytoplankton-mediated, and the effect of temperature was greatest for "away" phytoplankton treatments. I hypothesize that the enhanced effect of "away" phytoplankton on bacteria is due to the availability of a different pool of exudates than bacteria had become acclimated to in their "home" lake.

In Chapter 5, I describe a natural experiment in Emerald Lake that provides support for exudate-mediated phytoplankton effects and strengthens the evidence for shifts in dissolved organic matter (DOM) structuring bacterial communities. Emerald Lake is a high elevation lake where DOM is dominated by terrestrial sources in the spring, while phytoplankton-derived

autochthonous inputs become more important throughout the ice-free season. Glycolate-utilizing bacteria, a subset of the bacterial community able to use algal exudates, were characterized using DNA analysis of glycolate oxidase subunit D (*glcD*) genes from Emerald Lake samples collected approximately biweekly from ice-cover (June) through fall turnover (September). *glcD* genes were not detected in early season samples when the lake was ice-covered and phytoplankton-derived resources were scarce. Following this period, *glcD* gene composition exhibited significant changes through time, which were strongly correlated with the combination of fluorescence index, an indicator of the proportion of lake versus terrestrially derived DOM, and dissolved inorganic nitrate+nitrite. Chapter 5 was published in *FEMS Microbiology Ecology* in March 2013.

Summary

Phytoplankton seasonal succession effects concomitant changes in the composition of the total bacterial community and subtypes in the *Polynucleobacter* genus. My findings additionally provide insight into the mechanisms of phytoplankton interactions, which bacterial taxa are affected by phytoplankton, and how the influence of phytoplankton changes across different environmental contexts. This information will contribute to the ability to understand and predict patterns of bacterial community change, thereby providing a backdrop for examining mechanisms that create and maintain diversity and enhancing the ability to forecast community responses to environmental change.

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FIGURES

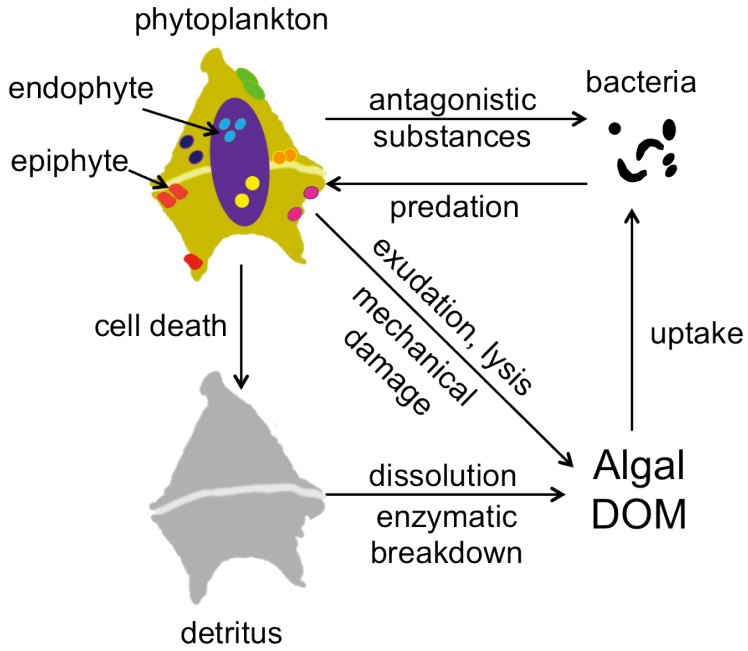

Figure 1.1. Mechanisms of phytoplankton effects on bacterial community composition adapted from Cole (1982).

CHAPTER 2

INTERACTIONS BETWEEN SPECIFIC PHYTOPLANKTON AND BACTERIA AFFECT LAKE BACTERIAL COMMUNITY SUCCESSION[1]

ABSTRACT

Time-series observations and a phytoplankton manipulation experiment were combined to test the hypothesis that phytoplankton succession effects changes in bacterial community composition. Three humic lakes were sampled weekly May-August over two years and correlations between abundances of specific phytoplankton and bacterial taxa in each time-series were determined. To experimentally characterize phytoplankton influence, bacteria from each lake were combined with phytoplankton from each lake or no phytoplankton. Following incubation, variation in bacterial community composition explained by phytoplankton treatment increased 65%, while the variation explained by bacterial source decreased 64%. Free-living bacteria explained, on average, over 60% of the difference between phytoplankton and corresponding no-phytoplankton control treatments. Fourteen out of the 101 bacterial taxa that exhibited positively correlated patterns of abundance with specific algal populations in time-series observations were enriched in mesocosms following incubation with phytoplankton and one out of 59 negatively correlated bacterial OTUs was depleted in phytoplankton treatments. Bacterial genera enriched in mesocosms containing specific phytoplankton assemblages included *Limnohabitans* (clade betI-A), *Bdellovibrio*, and *Mitsuaria*. These results suggest that effects

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Author contributions: SFP and ADK designed research. SFP and KRH conducted experiment. KRH carried out molecular analysis of experimental samples. AG, KAG, JRF, CTB, AGD-R, DBC, RR, and EWT generated Illumina sequence data and AGD-R carried out sequence classification. SFP and KRH carried out MDS and EDGE analyses. SFP carried out all other analyses and wrote the paper.

of phytoplankton on certain bacterial populations, including bacteria tracking seasonal changes in algal-derived organic matter, result in correlations between algal and bacterial community dynamics.

INTRODUCTION

Microbial contributions to energy flow and biogeochemical cycling are fundamentally important to aquatic ecosystem function. In particular, individual bacterial taxa have distinct combinations of metabolic capabilities (e.g. decomposition of specific carbon compounds, nitrogen transformations), such that alterations to bacterial community structure yield changes in rates of functions performed by bacteria (Bertilsson et al., 2007; Ishida et al., 2008; Strickland et al., 2009). Species interactions (e.g., competition, predation, facilitation) and environmental conditions that influence bacterial community composition and temporal dynamics therefore have potential implications for ecosystem function. In lakes, interactions between phytoplankton and bacteria have been proposed to influence pelagic bacterial community dynamics (Kent et al., 2007; Šimek et al., 2008; Paver and Kent, 2010; Paver et al., 2013). Establishing the effect of phytoplankton assemblages on bacterial community temporal patterns and investigating potential mechanisms of algal influence will provide insight into the ecology of microorganisms, as well as enhance the ability to predict microbial response to environmental change.

Effects of phytoplankton on lake bacterial community dynamics have been inferred from environmental observations and bacterial response to phytoplankton manipulation. Lake phytoplankton communities undergo seasonal succession due to factors that include changes in nutrient availability (e.g., silica, phosphorus, nitrogen) and grazing pressure (Sommer et al., 1986; Graham et al., 2004). Changes in phytoplankton community composition have been observed to correlate with changes in bacterial community composition (Kent et al., 2004; Kent

et al., 2007). In a study of six humic lakes, over half of the variation in bacterial community composition over a three-month period was explained by temporal patterns in the phytoplankton community and covariation between changes in phytoplankton and the environment (Kent et al., 2007). While correlated patterns suggest that phytoplankton influence bacterial community dynamics, they do not necessarily result from a causal relationship. Environmental time-series observations and experimental manipulations need to be combined to establish phytoplankton influence on bacterial community dynamics. For example, at the population level, bacteria within the genus *Limnohabitans* increased in abundance in a reservoir during periods dominated by cryptophytes (Šimek *et al*., 2008), and bacterial isolates from this genus exhibited enhanced growth when co-cultured with certain algae or amended with algal exudates (Šimek et al., 2011). A combined approach investigating community-level responses to phytoplankton has yet to be reported.

The influence of phytoplankton on bacterial community dynamics may be exerted through a number of potential taxon-specific mechanisms. Bacteria rapidly utilize exudates released by phytoplankton (e.g. sugars, amino acids) (Maurin et al., 1997; Sadro et al., 2011). The composition of phytoplankton exudates is species-specific (Fogg, 1983) and it has been shown that taxonomic composition of seawater bacterial cells taking up algal exudates and bacterial community composition following incubation with exudates depends on the phytoplankton species that produced the exudates (Sarmento and Gasol, 2012). Detritus following algal cell death is another source of phytoplankton-derived organic matter that has a species-specific effect on bacteria (van Hannen et al., 1999). Succession of the abundance and composition of genes associated with the utilization of algal-derived organic substrates, changes in enzyme expression, and changes in dissolved organic matter uptake patterns in freshwater and

marine systems lend further support to organic matter mediated influence of phytoplankton (Arrieta and Herndl, 2002; Lau and Armbrust, 2006; Lau et al., 2007; Paver and Kent, 2010; Eckert et al., 2012; Paver et al., 2013; Teeling et al., 2012). In addition to providing a source of organic matter, phytoplankton can provide a habitat to endophytic bacteria living within algal cells and epiphytic bacteria that live in the phycosphere surrounding algal cells (Cole, 1982). Marine bacteria in close association with phytoplankton differ in composition among algal cells of different species and are distinct from the free-living assemblage (Grossart et al., 2005; Jasti et al., 2005). Phytoplankton can also negatively affect populations within the bacterial community through nutrient competition, antibiotic release and bactivory by mixotrophs (Cole, 1982; Nygaard and Tobiesen, 1993).

Our objective was to determine whether taxon-specific interactions with phytoplankton contribute to temporal patterns in lake bacterial community composition. We selected three humic lakes where Kent and colleagues (2007) previously observed correlated phytoplankton and bacterial community dynamics as study sites. Local similarity analysis was used to detect bacterial operational taxonomic units (OTUs) whose relative abundances have been correlated to the abundance of certain populations of phytoplankton in weekly time-series observations from each lake. To experimentally assess the effect of phytoplankton on bacterial community composition, bacterial communities from each lake were subsequently incubated with the phytoplankton assemblage from one of the three lakes or no phytoplankton (control) in a seven-day mesocosm experiment. If interactions between specific phytoplankton and bacterial populations drive bacterial community dynamics, then: (i) correlated patterns of abundance between certain phytoplankton taxa and bacterial OTUs will be detected in environmental time-series observations, (ii) bacterial communities will diverge in composition when incubated with

different phytoplankton assemblages and initially distinct bacterial communities will become more similar when incubated with the same phytoplankton assemblage, and (iii) bacterial OTUs that respond to particular phytoplankton species, e.g. *Gymnodinium fuscum*, will increase (or decrease) in abundance when incubated with phytoplankton assemblages containing *G. fuscum* relative to no-phytoplankton control treatments and will exhibit a seasonal pattern of relative abundance that is positively (or negatively) correlated with *G. fuscum* abundance. After establishing the effect of phytoplankton assemblages on bacterial community composition, we used Illumina sequencing to determine which bacterial genera were enriched in phytoplankton treatments relative to no-phytoplankton control treatments.

RESULTS AND DISCUSSION

Environmental correlations between algal and bacterial OTUs

Over two years of weekly environmental observations between late May and mid-August in Crystal Bog (CB), South Sparkling Bog (SSB), and Trout Bog (TB), differences in dominant phytoplankton taxa were observed across lakes (Table 2.1). Some differences in phytoplankton taxa were also observed between years within the same lake, though to a lesser extent than among-lake differences (Table 2.1). Local similarity analysis identified 108 significant positive correlations and 60 significant negative correlations between the abundance of a specific algal taxon (characterized morphologically) and the relative abundance of a specific bacterial OTU (defined operationally by unique ARISA fragment length) in the six seasons of observation (three lakes, two years) (Fig. 2.1). Six pairs of algal and bacterial taxa were positively correlated and one algal-bacterial pair was negatively correlated in both years within a given lake. One algal-bacterial pair, *G. fuscum* and bacterial ARISA fragment 684, was significantly positively

correlated in SSB in 2003 and TB in 2008, and exhibited a generally positive relationship in five of the six environmental time-series (Fig. 2.2).

These observed taxon-level correlations support the hypothesis that interactions between specific phytoplankton and bacteria underlie observed correlations between phytoplankton and bacterial community dynamics (Kent et al., 2007). Correlations between the abundance of specific phytoplankton taxa and bacterial relative abundance, including a positive correlation between *G. fuscum* and ARISA fragment 684, have previously been detected in CB in 2002 (Newton et al., 2006). Two of the caveats to a correlational approach are that not all algal-bacterial interactions will be detected as correlations and that correlations do not necessarily result from interacting populations. Correlations between the abundance of *G. fuscum* and ARISA fragment 684 in time-series observations (Fig. 2.2) illustrate how some algal-bacterial pairs may be generally positively correlated, but not pass the significance threshold. Additionally, bacterial taxa may consume exudates derived from multiple phytoplankton species (Sarmento and Gasol, 2012), making it unlikely to detect correlation with specific phytoplankton species in a mixed community. Population dynamics can also be controlled by other factors such as grazers, viruses, and competing bacteria (Kent et al., 2006; Wei et al., 2011). Correlations do, however, provide an incomplete list of potentially interacting bacteria and phytoplankton that can then be tested experimentally.

Bacterial response to phytoplankton manipulation

Bacteria from each of our study lakes were combined with phytoplankton from one of the three lakes or no phytoplankton as a control. Initial bacterial community composition in each mesocosm was primarily explained by the source lake of the bacteria (CB, SSB, or TB) and

reflected a minor influence of the phytoplankton treatment (CB, SSB, TB, or control) (Table 2.2) (Fig. 2.3). Bacterial communities in mesocosms with bacteria from CB were similar in composition to those with bacteria from SSB and comparatively distinct from communities in mesocosms containing TB bacteria (average Bray-Curtis similarities: CB-SSB = 57%, CB-TB = 30%, SSB-TB = 32%). Similarly, CB and SSB phytoplankton assemblages were dominated by *Synura* and contained similar taxa while the phytoplankton assemblage from TB was distinct and contained taxa that were not detected or in low abundance in the other lakes including *Asterionella* sp.*, G. fuscum, and Mallomonas* sp. (Table 2.3).

Mesocosm microbial communities were contained in 10 L LDPE cubitainers and incubated at the surface in the center of Crystal Bog where temperatures averaged 21.8°C for seven days (range: 20.0-23.6°C; hourly temperature data from CB instrumented buoy: http://lter.limnology.wisc.edu/data/filter/10815). Following incubation, the variation in mesocosm bacterial community composition explained by phytoplankton treatment increased while the variation explained by bacterial source lake decreased (Table 2.2). Bacteria incubated with phytoplankton from CB or SSB developed similarly, whereas incubation with the TB phytoplankton assemblage had a unique effect on bacterial community composition (Fig. 2.3).

Shifts in bacterial community composition in response to incubation with various phytoplankton assemblages provide experimental evidence of phytoplankton assemblages shaping bacterial community development. This evidence supports the hypothesis that phytoplankton succession drives seasonal patterns in bacterial communities previously observed in these lakes (Kent et al., 2007; Paver and Kent, 2010). The similar effects of CB and SSB phytoplankton and unique effect of TB phytoplankton is likely due to the similarity of the major phytoplankton taxa in CB and SSB assemblages and the distinct assemblage characterized in TB

(Table 2.3). Observed changes in bacterial communities induced by the phytoplankton assemblage corroborate prior observations of the effect of phytoplankton on bacterial community development following incubation with detritus from different algal species in lake water mesocosms (van Hannen et al., 1999) and incubation with phytoplankton assemblages dominated by either diatoms or phytoflagellates in seawater mesocosms (Pinhassi et al., 2004).

To better understand how phytoplankton assemblages influenced bacterial communities, we classified bacterial OTUs according to whether they were abundant in the > 5 μm fraction of the community compared with the total community (> 0.22 μm) in each treatment as a proxy for phytoplankton attachment before and after incubation. We then determined what percentage of the post-incubation difference between each treatment and the corresponding no-phytoplankton control could be explained by bacterial OTUs in each category (Table 2.4). Free-living bacteria, those with higher relative abundance in > 0.22 μm sub-samples than > 5 μm sub-samples during the experiment, explained, on average, over 60% of the difference between treatments and the corresponding no-phytoplankton control. Phytoplankton-derived organic matter including exudates likely facilitated the growth of phytoplankton-enriched free-living taxa (Sarmento and Gasol). Free-living bacteria that were depleted in relative abundance in phytoplankton treatments compared with no-phytoplankton controls may have been directly negatively impacted by phytoplankton [e.g. predation by mixotrophs (Stoecker, 1999), antibiotics (Cole, 1982)] or indirectly negatively impacted by the proliferation of organisms with positive growth responses to phytoplankton. Depletion in relative abundance in phytoplankton treatments could potentially be an artifact of using relative abundance data; bacteria with constant or even slightly increasing population sizes may appear to decrease if other populations are increasing rapidly. Alternatively, in the absence of labile phytoplankton-derived substrates in control treatments,

phototrophic bacteria or bacteria that can utilize more recalcitrant carbon pools may have had a competitive advantage, explaining an increase in relative abundance in control treatments.

Another 5.1% of the difference in bacterial community composition between phytoplankton and control treatments was explained by bacterial OTUs that were enriched in phytoplankton treatments and presumed to have colonized algal cells during the experiment due to their appearance in the > 5 μm fraction of phytoplankton treatments following incubation. Phytoplankton-enriched free-living taxa consistently explained more of the differences between phytoplankton and control treatments than did phytoplankton-enriched colonizing bacteria, but the relative differentiating power of these groups varied depending on the specific treatment. Bacterial communities incubated with TB phytoplankton, which exhibited the most distinct community composition post-incubation (Fig. 2.3), were particularly strongly differentiated from control treatments by phytoplankton-enriched colonizing bacteria. Replicate observations of putative colonization of phytoplankton assemblages by specific bacterial OTUs can be explained by species-specificity between phytoplankton and their bacterial associates (Grossart et al., 2005; Jasti et al., 2005). Presumed phytoplankton-colonizing bacteria also potentially include bacteria that formed aggregates upon exposure to algal extracellular products. Micro-aggregate formation occurred when marine bacteria were incubated with dissolved organic carbon from the diatom *Chaetoceros* sp.; however, aggregate formation was determined to be atypical since it did not occur when bacteria were incubated dissolved organic carbon from five other phytoplankton species studied (Sarmento and Gasol, 2012).

Combining observational and experimental approaches at the OTU-level

Fourteen of the bacterial OTUs that were positively correlated with phytoplankton populations in environmental time-series observations were also enriched in mesocosms following incubation with phytoplankton relative to no-phytoplankton controls (Table 2.5). One bacterial OTU was negatively correlated with phytoplankton populations in the environment and also decreased in relative abundance following incubation with phytoplankton relative to no-phytoplankton controls. Most of these bacterial OTUs were categorized as free-living, but four were identified as potential phytoplankton colonists and ARISA fragment 684 was phytoplankton-associated throughout the experiment.

The observed correspondence between environmental and experimental results at the OTU-level supports the hypothesis that phytoplankton affect the dynamics of specific bacterial populations, thereby effecting change in bacterial community composition. Positive correlations between bacterial OTUs and phytoplankton were more numerous, repeatable between years, and overlapped to a greater degree with results from the mesocosm experiment than negative correlations, suggesting that facilitative effects of phytoplankton on bacteria are more important that inhibitory effects in contributing to bacterial community patterns in this system. While there were more free-living bacterial taxa that exhibited consistent responses to phytoplankton, phytoplankton-associated bacteria also appear to contribute to bacterial community dynamics. The relationship between phytoplankton-associated bacteria 684 and *G. fuscum* was the most consistent algal-bacterial relationship inferred from observations of these lakes (Fig. 2.2) (Newton et al., 2006). This consistency is likely due to bacteria 684 living in or on *G. fuscum* as suggested by the enrichment of bacteria 684 in the phytoplankton-associated fraction. Šimek and colleagues (2008; 2011) have also reported correspondence between observational and

experimental results. Bacteria within the genus *Limnohabitans* increased in abundance during periods dominated by cryptophytes (Šimek et al., 2008) and isolates from two lineages within this genus exhibited enhanced growth when co-cultured with certain phytoplankton including the cryptophyte *Cryptomonas* (Šimek et al., 2011). Taken together, the results from the current study and studies conducted by Šimek and colleagues demonstrate that phytoplankton affect the relative abundance of specific bacterial taxa and growth rate of bacterial strains and that these phytoplankton interactions scale up to effect seasonal patterns of change in bacterial communities.

Identification of bacterial taxa responding to phytoplankton manipulation

Illumina sequencing was used to identify bacterial taxa responding to phytoplankton treatments in the mesocosm experiment. Abundant bacterial clades in mesocosms, defined as those averaging greater than 1% of sequences across all treatments either before or after incubation, included representatives of the *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Verrucomicrobia* (Table 2.6). The two most abundant clades were Pnec and acI-B. The betaproteobacterial Pnec clade contains endosymbiotic as well as free-living representatives of the *Polynucleobacter* genus, which are ubiquitously detected in lakes and are ecologically diverse (Vannini et al., 2007; Jezbera et al., 2011; Newton et al., 2011). The second most abundant group of sequences at the clade level was acI-B. *Actinobacteria* within the acI lineage are also ubiquitous in freshwater lakes (Newton et al., 2007).

A subset of identified taxa that increased or decreased in relative abundance in phytoplankton treatments while remaining constant or exhibiting the opposite change in the

corresponding no-phytoplankton control (Fig. 2.4). Consistent enrichment of certain bacterial taxa was observed across bacterial communities originating from different lakes that were incubated with the phytoplankton assemblage from a particular lake. For example, the proportion of *Mitsuaria* sequences increased in all mesocosms containing SSB phytoplankton and *Bdellovibrio* increased in all mesocosms containing TB phytoplankton while not changing or decreasing in no-phytoplankton controls. Notably, about half of the taxa that were enriched or depleted in phytoplankton treatments could not be identified to lineage or clade by the system described by Netwon and colleagues (2011). Some of these identified genera are members of minor freshwater lake phyla, while others belong to well-described freshwater phyla, but families and genera that are not commonly detected in freshwater (Newton et al., 2011). Detection of taxa previously not detected in freshwater is likely a result of increased sequencing depth facilitated by highthroughput sequencing approaches. Eiler and colleagues (2012) characterized lake environmental time-series samples using 454 pyrosequencing of 16S rRNA and found that 38.3% of sequences could not be classified to the described freshwater tribes and clades. Phytoplankton enrichment of atypical lake bacterial taxa suggests that previously overlooked taxa may play significant ecological roles and contribute to temporal patterns of bacterial communities observed in lakes (Kent et al., 2007; Shade et al., 2007; Nelson, 2009).

Many of the phytoplankton-enriched bacterial taxa in this study include host-associated organisms, with a range of hosts that include phytoplankton. Phytoplankton enhanced growth has been observed for organisms within the *Limnohabitans* (clade betI-A) (Šimek et al., 2011) and *Campylobacter* (Axelsson-Olsson et al., 2010) genera. Additionally, *Acidovorax facilis* has been detected on diatom microaggregates (Knoll et al., 2001). Phytoplankton-enriched taxa also included a prevalence of genera associated with oral and intestinal flora including *Bacteroides*,

Capnocytophaga, *Eikenella*, *Eubacterium*, *Faecalibacterium*, *Fusobacterium*, *Gemella*, *Kingella*, *Leptotrichia*, *Neisseria*, *Porphyromonas*, *Prevotella*, *Selenomonas*, *Tannerella* and *Veillonella* (Christersson et al., 1991; Kamiya et al., 1993; Tyrrell et al., 2003; Rudney et al., 2005; Liu et al., 2008; Jia et al., 2010). Other genera enriched in these mesocosms include organisms previously associated with cockroaches [*Candidatus* Rhabdochlamydia (Corsaro et al., 2007)], amoeba [*Campylobacter* (Axelsson-Olsson et al., 2010)], lichen [*Methylobacterium* (Hodkinson and Lutzoni, 2009)] and plant roots [*Azohydromonas* and *Ideonella* (Coelho et al., 2008)]. Growth of enteric bacteria has previously been observed to coincide with the bloom of an algal mat in a pristine stream (McFeters et al., 1978). These coliform bacterial isolates were then demonstrated to grow on filter-sterilized extracellular products of *Chlorella*. While a bacterial genus may include a variety of organisms with distinct metabolic capabilities and ecological strategies, the prevalence of host-associated organisms with close relation to phytoplankton-enriched bacteria suggests that organisms within certain lineages have evolved to interact with a range of hosts.

While this study focused on demonstrating the effect of phytoplankton on bacterial community dynamics, bacterial communities also have the potential to affect phytoplankton community dynamics. Some genera enriched in phytoplankton treatments contain organisms with that negatively affect specific phytoplankton. *Acidovorax* contains a species capable algacidal activity (Kang et al., 2008), and both *Bdellovibrio* (Burnham et al., 1976) and *Achromobacter* (Wang et al., 2010) contain organisms that have an algalytic effect on specific cyanobacteria. Enrichment of certain bacterial taxa may have positive or negative feedback on the growth of particular phytoplankton similar to the framework developed for understanding interactions between plants and soil microorganisms (Bever et al., 2010).

Limitations

There are potential limitations in our study design that merit discussion. ARISA community fingerprinting was selected to characterize changes in OTU relative abundance across time-series observations and to link environmental and experimental observations. One drawback to ARISA fingerprinting is that some fragment lengths represent multiple, diverse taxa (Newton et al., 2006). Based on 16S rRNA + ITS clone libraries constructed from selected CB samples collected over a 3-year span, about 30% of ARISA fragment lengths were assigned to multiple taxa (Newton et al., 2006). For the majority of taxa in these lakes, using ARISA data to characterize population dynamics is a useful approach. ARISA has been shown to be comparable to a quantitative PCR approach in its ability to represent relative abundance of dominant *Candidatus* Accumulibacter clades (He et al., 2010). Strong correlations have also been observed between *Prochlorococcus* cell counts and abundance profiles from ARISA (Brown et al., 2005).

A second limitation with ARISA, which is also applicable to the 16S rRNA sequencing approach used here, is the use of relative abundance data. Since these relative abundance approaches are not quantitative, it is possible for an OTU to decrease in relative abundance while maintaining a consistent population size or even increasing in abundance if other populations are increasing more rapidly. Despite the potential to misclassify the responses of certain weakly responding OTUs, a relative abundance approach is useful for identification of the bacterial OTUs that are the most responsive to phytoplankton, and necessary for interpretation of observed patterns within the context of previous studies that have used relative abundance approaches (e.g. Kent et al., 2007; Shade et al., 2007; Nelson, 2009; Eiler et al., 2012). Additionally, based on previous experimental work conducted by Kent and colleagues (2006) in Crystal Bog using the same mesocosm containers and incubation time as the current study, bacterial abundance is

expected to remain relatively constant over time in the absence of added nutrients (Fig. A.1), lessening the potential for OTU response misclassification.

Phytoplankton collection for the mesocosm experiment using filtration made it possible to use native phytoplankton assemblages, but it also introduced potential confounding effects of including additional organisms. A small number of rotifers identified as *Keratella* sp. (0.1 ± 0.1 cells/ ml) were retained in the 20 to 100 μm size fraction. Rotifers are filter feeders known to ingest bacteria and smaller phytoplankton and may selectively graze bacteria >0.5 μm (Arndt, 1993), which could decrease certain bacterial and algal populations. In addition to rotifers, phytoplankton treatments also contained bacteria attached to phytoplankton. Phytoplankton-associated bacterial taxa that were higher in abundance in phytoplankton treatments than no-phytoplankton controls explained 6.7% of the difference between phytoplankton and no-phytoplankton treatments. While 6.7% is a not a negligible percentage, it does not invalidate the observation of phytoplankton effects on bacterial community composition and dynamics.

Effect of species interactions on planktonic community dynamics

Experimental and observational results from this study support the hypothesis that taxon-specific interactions with phytoplankton are an important ecological driver of bacterial community dynamics in lakes. Bacterial responses to phytoplankton appear to be primarily positive and to involve both loosely associated free-living bacteria and, to a lesser degree, bacteria closely associated with phytoplankton. Our sequencing results corroborate the observations of Šimek and colleagues (2008; 2011) that the betI-A clade, which encompasses the *Limnohabitans* genus, responds positively to incubation with phytoplankton and contributes to correlated patterns of algal and bacterial community change. Sequencing results also identified

additional taxa that warrant further investigation into their relationship with phytoplankton. From a community ecology perspective, this work provides an example of the importance of biotic drivers and potential for facilitation to structure communities. Future work is needed to determine how the magnitude of the effect of phytoplankton assemblages compares to other factors that influence temporal patterns of bacterial communities [e.g. temperature (Shade et al., 2007; Adams et al., 2010)], how the effect of phytoplankton on bacterial communities may be altered by interactions with the environment, and to characterize functional consequences of phytoplankton-induced shifts in bacterial communities.

EXPERIMENTAL PROCEDURES

Environmental observations

Integrated epilimnion samples were collected from Crystal Bog (CB), South Sparkling Bog (SSB), and Trout Bog (TB), three hydrologically isolated humic lakes in the Northern Highland Lake District of Wisconsin (Table 2.1). Sample collection occurred weekly from the end of May through August in 2003 and 2008 using PVC pipe integrated samplers as described by Kent and colleagues (2004). Microorganisms were collected onto 0.22 μm filters (Supor-200; Pall Gelman, East Hills, NY, USA) and frozen at -20°C. Subsamples were preserved in 2% glutaraldehyde for phytoplankton identification and enumeration. Total N, total P and DOC were determined biweekly according to standard protocols of the North Temperate Lakes Long Term Ecological Research project (http://lter.limnology.wisc.edu/research/ protocols/) (Kent et al., 2007).

Algal exchange mesocosm experiment

Bacterial communities from CB, SSB, and TB were incubated with native algal assemblages from CB, SSB, TB, or filter-sterilized and autoclaved CB water (as a no-phytoplankton control) in a full factorial design with three replicates of each treatment. Microorganisms were collected from CB, SSB, and TB on 22 July 2009 using an integrated epilimnion sampler (Kent et al., 2004). Bacterial communities from each lake were separated from larger aquatic organisms by filtration through a 1 μm Polycap AS cartridge filter (Whatman, Piscataway, NJ, USA). Native algal assemblages were collected by filtering through a 100 μm nylon mesh with filter sterilized and autoclaved CB water (Spectrum Laboratories, Rancho Dominguez, CA, USA) to remove zooplankton and collecting, then rinsing algal cells captured on a 20 μm nylon mesh (Spectrum Laboratories), which allowed smaller organisms such as heterotrophic nanoflagellates and bacteria to pass through. A small number of rotifers (0.1 ± 0.1 cells/ml) were also included in 20-100 μm size fractions. Phytoplankton collected on the 20μm mesh were resuspended in 0.2 μm filter-sterilized, autoclaved CB water, concentrating phytoplankton from 50 L of lake water to 3 L of sterilized water. All combinations of bacteria (5 L of 1 μm filtered water) from each lake and 0.3 L of concentrated phytoplankton from each lake or a no-phytoplankton control (0.3 L of 0.2 μm filter-sterilized and autoclaved CB water) were combined in 10L LDPE cubitainers (I-Chem, Rockwood, TN, USA) and incubated at the surface in the center of Crystal Bog for seven days. A 500 mL subsample was collected from each mesocosm on days 0, 3, and 7. A 50 mL subsample was preserved in 2% glutaraldehyde for phytoplankton enumeration. Microorganisms from 100 mL subsamples of each mesocosm were collected onto 0.22 μm filters (Supor-200; Pall Gelman, East Hills, NY, USA) for characterization of the whole community, and 5 μm Isopore filters (Millipore, Billerica, MA,

USA) for characterization of particle-associated organisms and frozen at -20°C. A 50 ml subsample was preserved in 2% glutaraldehyde for phytoplankton enumeration. A 2 ml subsample was observed under a Leica MZ 12.5 stereomicroscope at 50x magnification to confirm the presence of living phytoplankton.

Microbial community analysis

Dominant phytoplankton species were identified microscopically from glutaraldehyde-preserved pre-incubation samples and samples collected before and after a 7-day pilot experiment (Table A.1), and counts were transformed using biovolume estimates generated for species common to these lakes (Graham et al., 2004). DNA was extracted from filters using FastDNA Spin Kits (MP Biomedicals, Solon, OH, USA). 2003 time-series phytoplankton counts and DNA samples were the same as those in Kent and colleagues (2007). The bacterial community from environmental and experimental samples was characterized using Automated Ribosomal Intergeneic Spacer Analysis (ARISA) (Fisher and Triplett, 1999) using conditions described by Kent et al. (2007). The 16S-23S rRNA intergenic spacer region was PCR amplified from 20 ng of each DNA sample with 6-FAM labeled, universal 16S rRNA primer 1406F (5'-TGYACACACCGCCCGT-3') and 23SR (5'-GGGTTBCCCCATTCRG-3') targeting the bacterial 23S rRNA gene. The polymerase chain reaction contained 1 mM deoxynucleoside triphosphates (Promega, Madison, WI, USA), 0.4 μM of each primer, PCR buffer with 2.5 mM $MgCl_2$ (Idaho Technology Inc., Salt Lake City, Utah, USA) and 0.05 U μl^{-1} GoTaq (Promega). PCR cycles had an initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 35 s, 55°C for 45 s, and 72°C for 2 min, with a final extension carried out at 72°C for 2 min carried out in an Eppendorf MasterCycler Gradient (Eppendorf AG, Hamburg, Germany). Fluorescently

labeled ARISA PCR amplicons were combined with a custom 100–1250 bp Rhodamine X-labelled internal size standard (Bioventures, Murfreesboro, TN) and analysed by the Keck Center for Functional Genomics at the University of Illinois via denaturing capillary electrophoresis using an ABI 3730XL Genetic Analyser (Applied Biosystems, Carlsbad, California, USA).

Size calling, profile alignment, and grouping peaks into bins of operational taxonomic units were carried out using GeneMarker version 1.75 (SoftGenetics, State College, PA, USA). To include the maximum number of peaks while excluding background fluorescence, a threshold of 500 fluorescence units was used. ARISA fragments known to correspond to chloroplasts were removed from the analysis. The signal strength of each peak was normalized to account for run-to-run variations in signal detection by dividing the area of individual peaks by the total fluorescence (area) detected in each profile.

Illumina sequencing

High throughput Illumina sequencing of 16S rRNA genes was conducted on one representative replicate from each treatment sampled on day 0 and all three replicates sampled on day 7, except in two treatments where one mesocosm container was determined to leak following the experiment and the affected replicate was identified as an outlier. Illumina sequencing was conducted as described by Fagen and colleagues (2012). Briefly, the V4 region of the 16S rRNA gene was amplified from DNA samples using universal primers 515F and 860R (Caporaso et al., 2011) with an added barcode sequence and Illumina adapters. PCR cycles consisted of an initial denaturation at 94ºC for 3 min, followed by 20 cycles of 94ºC for 45 s, 50ºC for 30 s, and 65ºC for 90 s, and a final elongation step of 65ºC for 10 min. PCR products were purified using the Qiagen (Valencia, CA, USA) PCR purification kit according to the manufacturer's protocol.

Sequencing was conducted on an Illumina IIx (Illumina, Inc., CA, USA) with two 101 base pair, paired read cycles. Sequences were first quality trimmed using a modified version of Trim2 (Huang et al., 2003), and the first 11 bases of the primer region of each paired read were removed to prevent biases based on the degenerate bases in the primer sequences. A minimum read length of 70 was required after trimming, and only complete pairs were used for analysis. Sequences from the multiplexed Illumina run were sorted into their respective samples of origin based on their barcode sequences. The average number of reads per sample was 35,323 and ranged from 1,203 to 87,194 reads. The database used for 16S rRNA analysis was compiled by the McMahon and Bertilsson research groups and contained freshwater 16S rRNA sequences based on Ribosomal Database Project release 8.0 (Maidak et al., 2001), additional freshwater-specific sequences (Newton et al., 2011), and the 97% operational taxonomic unit Greengenes database dated 4 February 2011 (Werner et al., 2012). Freshwater 16S rRNA sequences were classified according to the lineage/clade/tribe naming convention proposed by Newton and colleagues (2011) while non-freshwater sequences retained the conventional family/genus/species naming scheme. The database was formatted using TaxCollector (github.com/audy/taxcollector) (Giongo et al., 2010) and sequences were compared with the database using CLC reference assembly version 3.1 using paired reads parameters and a global alignment. To classify the sequences, 98% of each sequence had to align to a reference in the database. An 80% similarity cut-off was applied at the phylum level and a 95% similarity cutoff was applied at the clade/genus level. Sequences were uploaded to MG-RAST (Meyer et al., 2008) [IDs: 4508634.3- 4508679.3].

Data analysis

Environmental time series observations were analyzed using local similarity analysis (LSA) and Pearson correlations to detect correlated phytoplankton and bacterial taxa from a specific lake in a given year (e.g. CB 2003) (Ruan et al., 2006). Significance of local similarity scores was based on 1000 permutations. Correlations with local similarity scores greater than 0.3 and p-values < 0.001 were considered significant (Shade et al., 2010). LSA, Pearson correlations and calculation of corresponding p- and q-values (calculated to adjust for multiple comparisons) were performed using the LSA code developed by Ruan and colleagues (2006) in the R statistical environment (R Development Core Team, 2010). LSA networks were visualized using Cytoscape v. 2.6.1 (Shannon et al., 2003). Correlated populations were then compared among the six time series (three lakes, two years) and to taxa enriched in algal addition mesocosm experiments.

Pairwise Bray-Curtis similarities were calculated for every combination of samples using ARISA relative fluorescence. Non-metric multidimensional scaling (MDS) was used to display Bray-Curtis similarities in multidimensional space using PRIMER version 6 (PRIMER-E Ltd, Plymouth Marine Laboratory, UK) (Clarke and Warwick, 2001). A two-way crossed (orthogonal) permutational analysis of variance (PERMANOVA) was used to test the effect of bacterial and phytoplankton treatments on mesocosm bacterial community composition before and after incubation. PERMANOVA is a non-parametric multivariate analysis of variance that generates p-values using permutations (Anderson, 2001; McArdle and Anderson, 2001). PERMANOVA tests were run using the adonis function from the vegan package (Oksanen et al., 2011) in the R statistical environment (R Development Core Team, 2010). The SIMPER analysis in PRIMER version 6 was used to determine taxa differentiating the > 5µm fraction from the

whole community (> 0.22 μm) for each treatment on days 0, 3, and 7 and each phytoplankton assemblage treatment from the corresponding negative control on days 0 and 7 (PRIMER-E Ltd, Plymouth Marine Laboratory, UK) (Clarke and Warwick, 2001).

Extraction of Differential Gene Expression (EDGE) software (version 1.1.291) was used to identify bacterial taxa displaying differential responses in phytoplankton treatments compared with the corresponding no-phytoplankton control (Storey et al., 2005; Leek et al., 2006). Analyses in the EDGE package were designed for identification of differentially expressed genes across treatments, and have previously been used to detect bacterial taxa with differential responses to incubation in epilimnion and hypolimnion lake layers (Shade et al., 2010). Time course differential analysis was used to compare each treatment with the corresponding no-phytoplankton control over time (Leek et al., 2006). An abundance table of Illumina sequence data classified at the genus/clade level was subsampled using daisychopper (http://www.genomics.ceh.ac.uk/GeneSwytch/) so that sequencing depth was equal for each mesocosm sample with the same bacterial source (Eiler et al., 2012). Unclassified sequences, determined as the number of sequences classified at the phylum level but not at the genus/clade level, were included in the subsampled table. Three replicates of each treatment on day 7 were compared with the initial community (day 0). For analysis of ARISA community fingerprints, relative fluorescence data from all replicates on days 0, 3, and 7 were used and replicate was specified as a covariate. EDGE analyses were run using default parameters of 1000 permutations and a significance threshold of $q < 0.10$. The q-value is the false discovery rate analogue of the p-value, which is calculated to account for multiple comparisons (Storey, 2002). Change in the abundance of subsampled Illumina sequences of bacterial taxa with significantly different response to phytoplankton and control treatments was visualized by creating a heatmap using the

gplots package in R (R Development Core Team, 2010). To be included in the heatmap, each clade or genus had to be detected in at least two treatments on day 7, exhibit significant differences from the corresponding control in at least two treatments as determined by EDGE, and be classified as enriched or depleted in at least one phytoplankton treatment. Enriched and depleted taxa were defined as those that increased or decreased ($|change| > 0.005$), respectively, in relative abundance in at least one phytoplankton treatment while exhibiting the opposite change in the corresponding negative control.

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TABLES

Table 2.1. Lake characteristics during environmental time series sampling in 2003 and 2008

Lake	Latitude Longitude	Max depth	Surface area (Ha)	pH	Total N (µg/l)	Total P (µg/l)	DOC (mg/l)	Phytoplankton taxa with highest biovolume
Crystal Bog	46°00'26.8"N	2.5	0.56	5.1	629 (261)[a]	22 (7)	9.5	*Synura, P. limbatum, P. umbonatum, P. cinctum*
	89°36'22.5"W				623 (221)[b]	28 (23)	18.2 (14.6)	*P. umbonatum, G. fuscum, Synura, P. limbatum*
South Sparkling Bog	46°00'13.6"N	8.0	0.44	5.1	642 (161)[a]	22 (20)	11.2	*P. limbatum, Synura, Mallomonas, G. fuscum*
	89°42'19.9"W				585 (371)[b]	40 (46)	15.4 (14.4)	*P. limbatum, G. fuscum, Synura, Trachelomonas*
Trout Bog	46°02'27.5"N	7.9	1.01	4.8	815 (237)[a]	32 (22)	28.0	*G. fuscum, Asterionella, Mallomonas, Cryptomonas*
	89°41'09.6"W				692 (314)[b]	50 (115)	23.9 (16.9)	*G. fuscum, Cryptomonas, Gloeocystis, Asterionella*

Abbreviations: total N, total nitrogen; total P, total phosphorus; DOC, dissolved organic carbon; *P., Peridinium*; *G.*, *Gymnodinium.*
Numbers in parentheses represent the range of observed values (maximum value-minimum value)
2003 data from Kent et al. (2007)
[a]First value in each cell is from 2003, [b]second value in each cell is from 2008.

Table 2.2. Results from PERMANOVA testing the effect of bacterial source lake (CB, SSB, TB) and phytoplankton treatment (CB, SSB, TB, control) on bacterial community composition before (Day 0) and after (Day 7) incubation.

		df	Sum of Squares	Pseudo-*F*	*P*	R^2
Day 0						
	Bacteria	2	3.9	263.8	<0.001	0.84
	Phytoplankton	3	0.8	36.7	<0.001	0.17
	Bacteria*Phytoplankton	6	-0.2	-5.0	1.000	-0.05
	Residuals	24	0.2			0.04
Day 7						
	Bacteria	2	2.0	13.0	<0.001	0.30
	Phytoplankton	3	1.8	8.0	<0.001	0.28
	Bacteria*Phytoplankton	6	0.9	2.0	<0.002	0.14
	Residuals	24	1.8			0.28

Table 2.3. Phytoplankton biovolume from mesocosms assembled with phytoplankton from each lake at the start of the incubation ($\mu m^3 ml^{-1} \pm SE$).

	Asterionella	*Cryptomonas*	*Dinobryon*	*G. fuscum*	*Gloeocystis*	*Mallomonas*	*P. cinctum*	*P. limbatum*	*Synura*
CB	-	$2.1 \pm 1.3 \times 10^4$	$5.5 \pm 2.4 \times 10^2$	-	-	$4.2 \pm 4.2 \times 10^2$	-	$3.4 \pm 1.6 \times 10^5$	$1.6 \pm 0.3 \times 10^7$
SSB	-	$9.6 \pm 2.3 \times 10^3$	$1.7 \pm 0.8 \times 10^3$	-	-	-	$8.6 \pm 1.8 \times 10^4$	$6.1 \pm 1.4 \times 10^6$	$7.9 \pm 0.8 \times 10^6$
TB	$3.1 \pm 0.9 \times 10^3$	$7.8 \pm 0.9 \times 10^3$	$2.5 \pm 0.6 \times 10^3$	$3.8 \pm 0.8 \times 10^5$	$1.0 \pm 0.7 \times 10^3$	$1.2 \pm 0.1 \times 10^5$	$4.5 \pm 4.5 \times 10^3$	$1.3 \pm 1.0 \times 10^5$	$1.1 \pm 1.1 \times 10^4$

Abbreviations: *G. fuscum*, *Gymnodinium fuscum*; *P. cinctum*, *Peridinium cinctum*; *P. limbatum*, *Peridinium limbatum*
Oocystis ($2.1 \pm 2.1 \times 10^2$) was detected in CB, *Euglena* ($4.7 \pm 4.7 \times 10^2$) was detected in TB.

Table 2.4. Percent of the difference between each phytoplankton treatment and the corresponding no-phytoplankton control explained by phytoplankton-enriched or phytoplankton-depleted taxa categorized by their association with phytoplankton as determined by SIMPER analysis.

	CB phytoplankton			SSB phytoplankton			TB phytoplankton			Avg.
Bacterial source	CB	SSB	TB	CB	SSB	TB	CB	SSB	TB	
Phytoplankton-enriched										
Phyto-associated	18.7	8.0	8.6	2.4	6.2	3.8	4.0	8.3	0.2	6.7
Phyto-colonizer	8.4	2.6	3.2	0.6	0.1	1.0	6.9	13.9	9.0	5.1
Particle-associated	0.0	1.5	9.1	0.0	0.0	1.9	3.4	5.6	18.5	4.4
Free-living	17.0	31.4	34.6	50.5	36.9	48.0	29.1	24.6	23.8	32.9
Other	0.0	0.0	0.0	0.0	1.9	0.0	0.1	0.0	0.1	0.2
Phytoplankton-depleted										
Phyto-associated	3.7	5.5	0.0	5.8	7.1	0.2	5.7	4.3	1.2	3.7
Phyto-colonizer	0.3	1.7	0.0	8.3	1.7	5.1	1.1	0.6	15.8	3.8
Particle-associated	19.2	4.4	9.6	5.0	2.1	6.9	7.6	2.6	4.1	6.8
Free-living	25.0	35.4	30.7	20.2	32.9	25.8	37.2	32.0	21.6	29.0
Other	7.8	9.6	4.2	7.2	11.2	7.3	5.1	8.2	5.7	7.4

Definitions: Phyto-associated, higher in relative abundance in the >5µm sub-sample compared to the >0.22µm sub-sample on day 0; Phyto-colonizer, no difference in relative abundance on day 0 and higher in relative abundance in the >5µm sub-sample on day 7; Particle-associated, higher in relative abundance in the >5µm sub-sample in both phytoplankton treatment and no-phytoplankton control on day 7; free-living, higher in abundance in the >0.22µm sub-sample relative to the >5 µm sub-sample; other, no difference in abundance detected between >5µm and >0.22µm sub-samples of phytoplankton treatments.

Table 2.5. Bacterial taxa, defined by ARISA fragment length (AFL), that significantly increased (+) or decreased (-) over the mesocosm incubation period in phytoplankton treatments relative to no-phytoplankton controls and whose abundance was positively (+) or negatively (-) correlated with abundance of specific phytoplankton in one or more weekly environmental time series observations of CB, SSB, and TB from May-August 2003 and 2008.

	Mesocosm Experiment: Phytoplankton and Bacterial Treatments									Environmental Time Series: Phytoplankton Species												
	CB Phytoplankton			SSB Phytoplankton			TB Phytoplankton															
AFL	CB	SSB	TB	CB	SSB	TB	CB	SSB	TB	*Crucigenia*	*Cyclotella*	*Cryptomonas*^{CST}	*Dinobryon*^{CST}	*Gloecystis*^{T}	*G. fuscum*^{T}	*Mallomonas*$^{(C)T}$	*Oocystis*$^{(C)}$	*P. cinctum*$^{S(T)}$	*P. limatum*^{CST}	*P. umbonatum*	*Scenedesmus*	*Synura*$^{CS(T)}$	*Tabullaria*
389	+*		+*													+							
494				+									+		-			-	=				
548				+						+							+			-		+	
558			+									+	-									+	
565			+														+		-			‡	
598		-		+				-		+	+	+						+		-			
627									-*							+							
684								+^	+#						‡								
744				+														+	+				
749				+													+						
768			+																-				
805								+	+*														+
821									+#		-		+				-		-				
881		+		+				+	+			+		+		+	-		-			-	
891		+														-	-						
910			+				+		+		-		+			-	-						
917								-								+				+			
954			+	+												+	-				+	-	
988							+*		+#								-						

^ Phytoplankton-associated
* Phytoplankton-colonizer
# Particle-associated
C,S,T species was detected in phytoplankton assemblage from CB, SSB, and TB, respectively
( ) detection in mesocosm phytoplankton assemblage at low abundance
‡ positive correlation detected multiple times
= negative correlation detected multiple times
Abbreviations: CB, Crystal Bog; SSB, South Sparkling Bog; TB, Trout Bog; *G. fuscum*, *Gymnodinium*; *P. cinctum, P. limbatum* and *P. umbonatum*, *Peridinium*; *P. umb. umb.*, *Peridinium umbonatum umbonatum;*

Table 2.6. Average relative abundance before (1 replicate/ treatment) and after (3 replicates/ treatment) incubation of the most abundant bacterial clades detected in mesocosms. Clade classification and corresponding Linnaean classification to the most resolved level available is based on Newton et al. (2011).

Phylum	Clade	Linnaean classification	Average relative abundance	
			Day 0	Day 7
Alpha-proteobacteria	alfI-A	*Rhizobiales*	0.005	0.021
	alfIV-A	*Novosphingobium*	0.001	0.024
Beta-proteobacteria	betIII-A	*Alcaligenaceae*	0.040	0.062
	Pnec	*Polynucleobacter*	0.269	0.126
Gamma-proteobacteria	gamIII-A	*Acinetobacter*	0.002	0.010
Verrucomicrobia	verI-A	*Xiphinematobacter*	0.027	0.007
Actinobacteria	acI-A	*Actinomycetales*	0.011	0.001
	acI-B	*Actinomycetales*	0.207	0.039
	acV-A	*Acidomicrobiales*	0.033	0.001
Bacteroidetes	bacI-A	*Sediminibacterium*	0.004	0.021
	bacVI-A	*Mucilaginibacter*	0.004	0.044

FIGURES

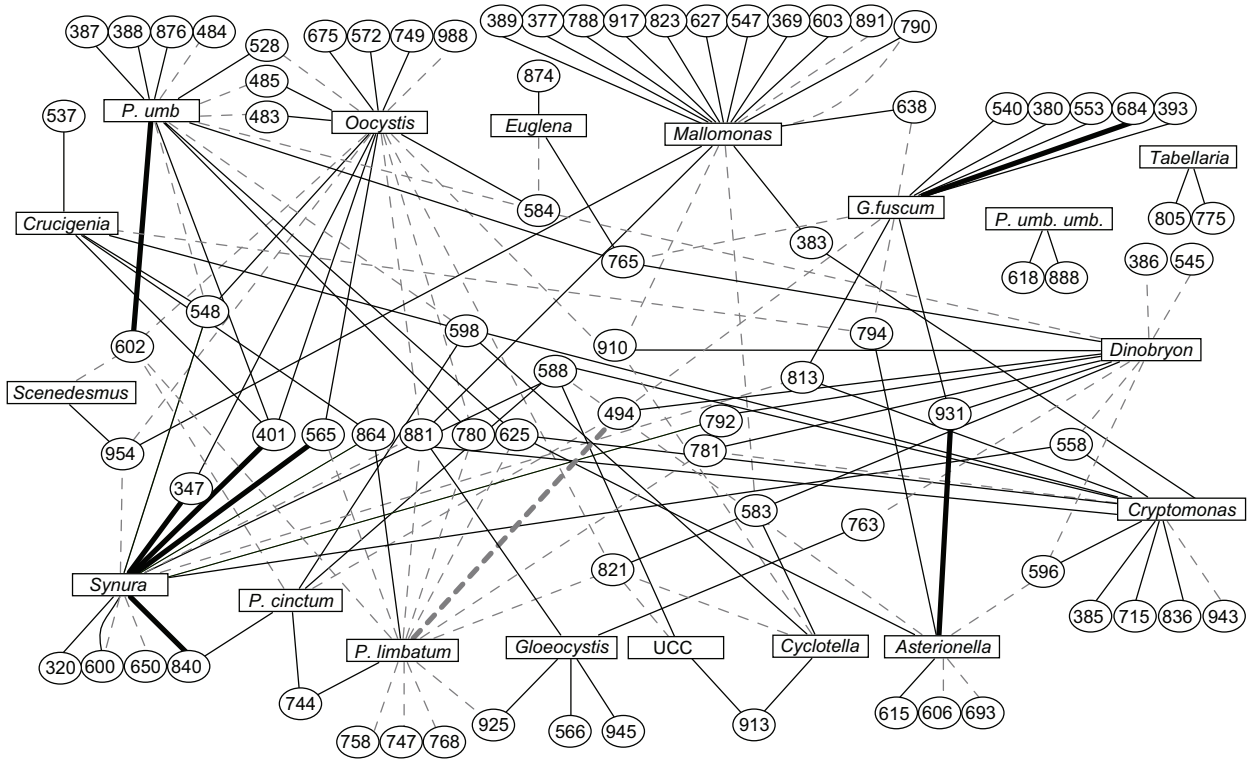

Figure 2.1. Network of all positive (solid lines) and negative (dotted lines) correlations between algal and bacterial taxa detected in CB, SSB, and TB in 2003 and 2008. Thick lines indicate correlations observed in two seasons (e.g. CB 2003 and CB 2008, TB 2003 and SSB 2008).

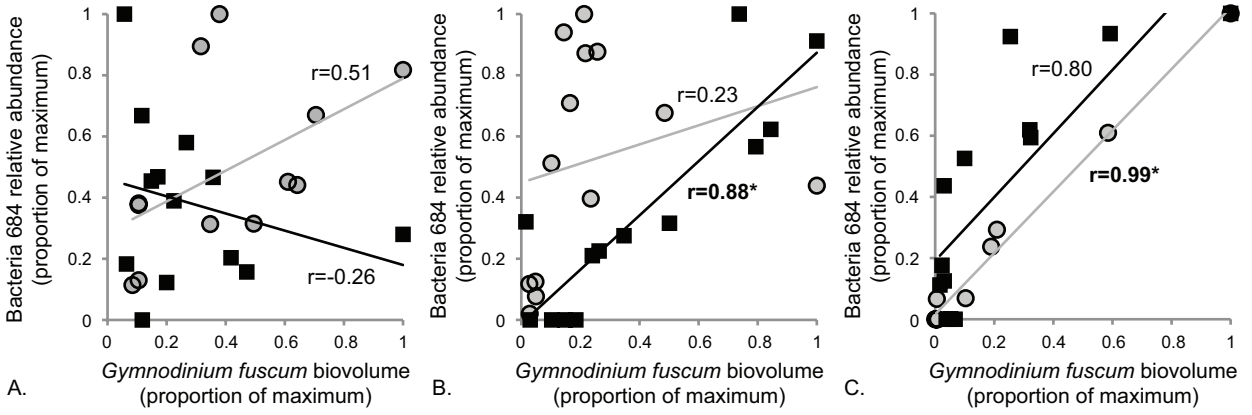

Figure 2.2. Relative abundance of bacterial OTU 684 as a function of the biovolume of phytoplankton *Gymnodinium fuscum* in CB (A), SSB (B), and TB (C) in 2003 (black squares) and 2008 (grey circles). Statistically significant r values according to local similarity analysis are indicated by bold font and an asterick.

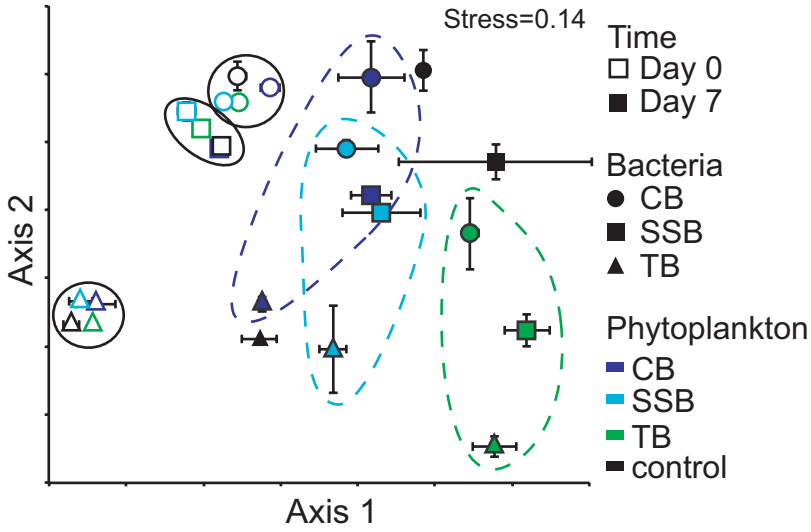

Figure 2.3. Nonmetric multidimensional scaling ordination of bacterial communities from Crystal (CB), South Sparkling (SSB) and Trout (TB) Bogs incubated with phytoplankton from CB, SSB, TB or a no-phytoplankton control before (Day 0 – open symbols) and after incubation (Day 7 – filled symbols). Symbols represent the average bacterial community in three replicate mesocosms observed using ARISA community fingerprinting, and bars indicate standard error along each axis. Shape indicates bacterial source, color indicates source of the phytoplankton treatment. Before incubation, bacterial community similarity was determined by bacterial source, as illustrated by solid ellipses. Following incubation, the phytoplankton source had a pronounced effect on bacterial community composition as illustrated with dotted ellipses.

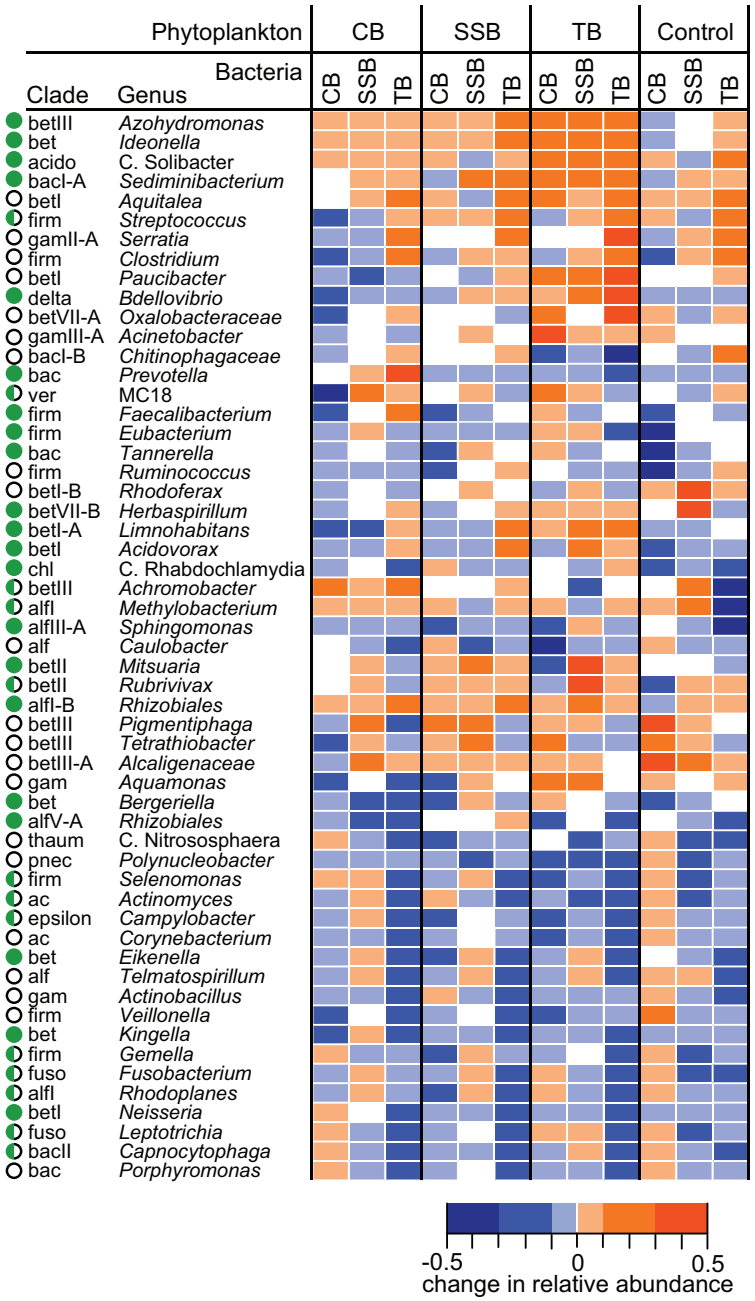

Figure 2.4. Change in relative abundance over the seven-day incubation (final relative abundance –initial relative abundance) of bacterial taxa significantly enriched or depleted in at least one phytoplankton treatment (CB, SSB, TB) relative to the corresponding no-phytoplankton control (control) based on EDGE analysis of Illumina sequencing data. Circles indicate whether each taxon was enriched by phytoplankton (green), depleted by phytoplankton (white), or both (half and half). Taxa were identified to clade using the naming scheme described by Newton et al. (2011) and genus using Linnaean classification. Where clade or genus names were not available, taxa were identified to lineage or phylum and family, respectively. Magnitude of change has been relativized across treatments for each taxon. Order of taxa is based on complete-linkage clustering. Abbreviations: C., Candidatus; alf, *Alphaproteobacteria*; bet, *Betaproteobacteria*; gam, *Gammaproteobacteria*; delta, *Deltaproteobacteria*; epsilon, *Epsilonproteobacteria*; ac, *Actinobacteria*; acido, *Acidobacteria*; bac, *Bacteroidetes*; chl, *Chlamydiae*; firm, *Firmicutes*; fuso, *Fusobacteria*; thaum, *Thaumarchaeota*; ver, *Verrucomicrobia*.

CHAPTER 3

PHYTOPLANKTON SUCCESSION AFFECTS THE COMPOSITION OF *POLYNUCLEOBACTER* SUBTYPES IN HUMIC LAKES

ABSTRACT

Interactions with phytoplankton influence the composition of bacterial communities, but the taxonomic specificity of these interactions is unclear due to the aggregation of ecologically distinct populations by community characterization methods. Here we examine whether phytoplankton seasonal succession affects the composition of subtypes within the cosmopolitan freshwater bacterial genus *Polynucleobacter*. Changes in the composition of *Polynucleobacter* subtypes were characterized in samples collected weekly May-August in 2003 and 2008 from three humic lakes using terminal restriction fragment length polymorphism fingerprinting of the protein-encoding cytochrome c oxidase *ccoN* gene. On average, 30% of temporal variation in the composition of *Polynucleobacter* subtypes was explained by changes in phytoplankton population abundances, with an additional 32% explained by changes in environmental factors and 18% by the interaction between phytoplankton and the environment. The effect of phytoplankton on specific *Polynucleobacter* subtypes was experimentally confirmed by changes in *Polynucleobacter* subtype composition observed in response to incubations with different phytoplankton assemblages or a no phytoplankton control. Phytoplankton-associated subtypes and differentiation in substrate use among subtypes likely contribute to the effects of phytoplankton on *Polynucleobacter* subtype composition. Interactions between unique

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Author contributions: The study was designed by S. F. Paver, R. J. Whitaker, and A. D. Kent. N. D. Youngblut generated *ccoN* gene sequence data, performed initial alignment, classified sequences into OTUs, calculated *Fst* values, and assisted with enzyme selection for T-RFLP. S. F. Paver performed all other analyses and wrote the paper.

Polynucleobacter populations and phytoplankton highlight the ecological significance and specificity of species interactions in freshwater communities.

INTRODUCTION

Species interactions can define the diversity, assembly, and succession of all communities including those that are microbial (e.g., Boenigk et al., 2004; Middelboe et al., 2009; Šimek et al., 2010; Luo et al., 2013; Paver et al., 2013b). Phylogenetically closely related aquatic bacterial strains (≥99.1% 16S rRNA gene sequence similarity) have been demonstrated to differ in susceptibility to grazing (Boenigk et al., 2004; Šimek et al., 2010) and viral lysis (Middelboe et al., 2009; Šimek et al., 2010) in laboratory experiments. Additionally, resource-based interactions (e.g., phytoplankton exudates) may promote substrate specialization like that observed among microdiverse freshwater bacterial isolates with identical 16S rRNA gene sequences (Jaspers and Overmann, 2004; Jogler et al., 2011). Despite the ecological importance of bacterial microdiversity, studies investigating the effects of species interactions on bacterial communities in the environment typically use characterization methods that can aggregate closely related, but ecologically distinct organisms (e.g., Jasti et al., 2005; Kent et al., 2006; Eckert et al., 2012; Paver et al., 2013b). The contribution of species interactions to the diversity and temporal patterns of closely related bacteria has implications for understanding the composition of microbial communities and how diversity is created and maintained in the environment.

Interactions between phytoplankton and bacterioplankton likely contribute to the diversity and temporal patterns of microdiverse bacterial populations. During the ice-free period in lakes, the abundance and composition of phytoplankton change in an annually repeated

process of seasonal succession (Sommer et al., 2012). Through interactions between specific phytoplankton and bacterial populations, phytoplankton seasonal succession induces changes in the composition of bacterial communities (Kent et al., 2007; Paver et al., 2013b). Phytoplankton may affect the abundance of bacterial populations through mechanisms such as selective grazing by mixotrophic phytoplankton (Flynn et al., 2013), serving as a habitat (Jasti et al., 2005), and providing a source of organic matter (Cole, 1982). There is considerable support for resource-mediated phytoplankton effects as bacterial genes and enzymes involved with utilization of algal resources have been demonstrated to change in response to phytoplankton blooms and seasonal succession across a range of aquatic ecosystems (e.g., Arrieta and Herndl, 2002; Lau et al., 2007; Paver and Kent, 2010; Teeling et al., 2012; Paver et al., 2013a). Phytoplankton resources, including exudates released by living cells and detritus, differ in chemical composition depending on the phytoplankton species (Kreger, 1962; Hellebust, 1965; Fogg, 1983) and alter bacterial community composition in experimental incubations by selectively enriching for specific bacterial taxa (van Hannen et al., 1999; Sarmento and Gasol, 2012). Substrate specialization has previously been observed among *Brevundimonas alba* strains with identical 16S rRNA gene sequences (Jaspers and Overmann, 2004) and *Sphingomonodaceae* isolates within the same internal transcribed spacer subgroup (Jogler et al., 2011).

Partitioning of phytoplankton resources may contribute to ecological diversification within the freshwater bacterial genus *Polynucleobacter*. *P. necessarius* ssp. *asymbioticus* (PnecC) is ubiquitously detected in freshwater habitats and is especially abundant in humic lakes where it can reach abundances of up to 67% of bacterial cells (Jezberová et al., 2010). Genetic analysis of 176 cultured strains of *P. n. asymbioticus* suggests that this subspecies is highly diverse (~300 and 800 genotypes estimated for 16S-23S ITS and *glnA* genes, respectively) and

that the diversity is underestimated using the 16S rRNA gene (~20 estimated genotypes) (Hahn et al., 2012). *P. n. asymbioticus* subgroups have different preferences with respect to pH/conductivity, altitude/surface area, and concentrations of dissolved organic carbon and oxygen that result in complete habitat separation between certain groups (Jezbera et al., 2011). Substrate utilization profiles of *Polynucleobacter* isolates differ within and among the four *Polynucleobacter* subgroups (PnecA, PnecB, PnecC, PnecD) with no coherent subgroup-specific differences in substrates utilized (Jezbera et al., 2012). Use of phytoplankton resources by *Polynucleobacter* populations has been inferred based on the abundance of *Polynucleobacter*-like sequences in freshwater surveys of bacteria with genetic potential to use the algal exudate glycolate (Paver and Kent, 2010; Paver et al., 2013a). However, the presence of a gene does not necessarily mean that the corresponding enzyme is being produced and some members of the *Polynucleobacter* genus do not appear to benefit from phytoplankton-derived resources (Hahn et al., 2012). Together these observations suggest that *Polynucleobacter* responses to phytoplankton dissolved organic matter are subtype-specific.

Our objectives were to characterize temporal patterns in the composition of *Polynucleobacter* subtypes within the epilimnion of humic lakes and determine whether any observed ecological differentiation could be attributed to interactions with phytoplankton. To achieve fine-scale phylogenetic resolution of *Polynucleobacter* subtypes, we characterized allelic diversity of the protein-encoding gene cytochrome c oxidase, cbb3-type, subunit I (*ccoN*) using *Polynucleobacter*-specific primers (Youngblut et al., 2013). Effects of lake, year, and environmental factors on *Polynucleobacter* subtype composition were determined in order to elucidate how the influence of phytoplankton acts in combination with other ecological drivers. If phytoplankton differentially affect *Polynucleobacter* subtypes, then (1) temporal patterns in

Polynucleobacter subtype composition will be explained by changes in phytoplankton composition and (2) *Polynucleobacter* subtype composition following incubation in experimental mesocosms will depend on the presence and composition of phytoplankton. To test our first prediction, *Polynucleobacter* subtype composition was characterized in 2003 and 2008 May through August time-series samples collected from Crystal Bog (CB), South Sparkling Bog (SSB) and Trout Bog (TB), three north temperate humic lakes where the *Polynucleobacter* PnecC subgroup is abundant and phytoplankton seasonal succession has previously been demonstrated to effect changes in bacterial community composition (Paver et al., 2013b; Youngblut et al., 2013). To test our second prediction, *Polynucleobacter* subtype composition was characterized before and after incubating bacteria from SSB and TB with phytoplankton from SSB, phytoplankton from TB, or no phytoplankton as a control.

RESULTS

Temporal patterns and environmental drivers of Polynucleobacter *composition*

Polynucleobacter ccoN gene sequences from selected CB, SSB, and TB environmental samples were classified into 51 operational taxonomic units (OTUs) (Fig. 3.1, Fig. B.1). OTUs were defined at the >99.5% similarity level to maximize OTU detection while mitigating the potential for novel OTUs to arise from sequencing errors. To date, the only *ccoN* gene sequences generated from characterized *Polynucleobacter* isolates are from the PnecC subgroup (Meincke et al., 2012; Hao et al., 2013), and these PnecC *ccoN* sequences are interspersed among our environmental *ccoN* sequences in a maximum likelihood tree (Fig. 3.1). The composition of *ccoN* OTUs differed spatially among lakes as well as over time within the same lake. Considering all *Polynucleobacter* gene sequences from the same lake and time (e.g., CB 14 July

2003) as a population, *Polynucleobacter* population differentiation was calculated using the fixation index (F_{ST}). Significant genetic differentiation was observed between certain samples collected at different times from the same lake (Table 3.1).

A terminal restriction fragment length polymorphism (T-RFLP) protocol was designed from *Polynucleobacter ccoN* sequences to characterize *Polynucleobacter* subtype composition in weekly samples collected from May through August in each of the three lakes in 2003 and 2008. Differences in subtype composition were observed among lakes and between years (PERMANOVA: Lake R^2=0.21, $p<0.001$; Year R^2=0.26, $p<0.001$)(Fig. B.2). Each lake had a characteristic *Polynucleobacter* assemblage that was maintained through time. Between May and August in each time series, we observed temporal changes in *Polynucleobacter* subtype composition similar to compositional changes seen for the phytoplankton assemblage over the same time period (Kent et al., 2007) (Fig. B.3). Approximately 30% of the seasonal variation in *Polynucleobacter* subtype composition was explained by changes in the abundance of phytoplankton populations (Fig. 3.2). The environmental factors and the interaction between phytoplankton populations and the environment explained an additional 32% and 18% of *Polynucleobacter* subtype variation, respectively.

Experimental effects of phytoplankton assemblages on Polynucleobacter *composition*

An algal-exchange experiment was conducted to directly test the effect of phytoplankton on *Polynucleobacter* subtype composition. *Polynucleobacter* composition was characterized before and after a five-day incubation where bacteria from SSB and TB were combined pairwise with phytoplankton from one of two lakes or no phytoplankton as a control. The phytoplankton assemblage from SSB was dominated by *Peridinium limbatum*, while *Cyclotella* dominated the

phytoplankton assemblage from TB. Differences in composition of *Polynucleobacter* subtypes in experimental mesocosms were explained by phytoplankton treatment, and the importance of phytoplankton treatment increased over the incubation period (Fig. 3.3, Table 3.2). Before incubation, there were observable phytoplankton treatment effects, indicating that some *Polynucleobacter* subtypes were closely associated with phytoplankton. Following incubation, *Polynucleobacter* composition was more similar to their initial composition in treatments without phytoplankton than in treatments with phytoplankton. The effect of TB phytoplankton on the composition of *Polynucleobacter* subtypes was especially pronounced.

Differences in composition of *Polynucleobacter* assemblages following incubation with phytoplankton treatments may be due to amplification of initial differences between phytoplankton and control mesocosms or shifts in composition during the incubation period. Amplification of initial differences is indicated by *Polynucleobacter* restriction fragments (T-RFs) that were enriched or depleted in phytoplankton treatments compared to no-phytoplankton controls both before and after incubation, termed here "consistently enriched" and "consistently depleted". Differences that arose during the incubation period are indicated by T-RFs that were only enriched or depleted following incubation, termed here "enriched following incubation" and "depleted following incubation". The percent of the difference between each phytoplankton treatment and the corresponding control following incubation explained by each T-RF was calculated using SIMPER analysis (Clarke and Warwick, 2001) and summed for T-RFs in each of the four categories (Table 3.3). Post-incubation differences between phytoplankton and control treatments were due, in part, to an amplification of initial differences. However, a substantial portion of variation was explained by T-RFs with no initial difference in relative

abundance that were enriched (avg. 17.6% variation) or depleted (avg. 16.0% variation) following incubation with phytoplankton.

Consistency of Polynucleobacter *responses to phytoplankton and potential for indirect effects*

To interpret the experimental results within their environmental context, we identified "phytoplankton-responsive" *Polynucleobacter* T-RFs as those that were significantly enriched or depleted in phytoplankton treatments compared to the corresponding control treatments in the experiment and also exhibited relative abundance patterns that were correlated with the abundances of specific phytoplankton populations in each time series (e.g., CB 2003). We used EDGE analysis for the experimental dataset to identify T-RFs responding differently to phytoplankton treatments and corresponding controls (Storey et al., 2005; Leek et al., 2006). For the environmental dataset, we used local similarity analysis to identify *Polynucleobacter* T-RFs whose relative abundance was positively or negatively correlated with the abundance of specific phytoplankton populations (Ruan et al., 2006). Of the 36 *Polynucleobacter* T-RFs positively correlated with phytoplankton populations in the environment six were enriched in relative abundance following incubation with phytoplankton in the experiment. Of the 24 *Polynucleobacter* T-RFs negatively correlated with phytoplankton populations in the environment two were depleted in relative abundance when incubated with phytoplankton in the experiment (Table B.1). Overall, consistency of responses to phytoplankton between the experiment and environment was low. Correlations between specific *Polynucleobacter* T-RFs and phytoplankton species were not observed in multiple time series (e.g., CB 2003 and CB 2008). Eight of the T-RFs that had inferred relationships to phytoplankton in the environment

and in the experiment had positive responses in the experiment and negative responses in the environment, or vice versa.

For *Polynucleobacter* subtypes that are affected by phytoplankton, the influence of phytoplankton can be (1) direct, (2) mediated by interaction with bacteria, or (3) mediated by interaction with another *Polynucleobacter* subtype (Fig. 3.4a). To determine whether bacteria mediate the consistent responses of certain *Polynucleobacter* subtypes to phytoplankton, local similarity analysis was used to detect correlations between abundances of phytoplankton, bacterial, and *Polynucleobacter* populations in the environment. If correlations were detected among a given phytoplankton-responsive *Polynucleobacter* T-RF, bacterial ARISA fragment, and phytoplankton species, then patterns in relative abundance were compared for the bacterial ARISA fragment and *Polynucleobacter* T-RF in response to incubation with phytoplankton in the algal exchange experiment. *Polynucleobacter* T-RFs HR_826 and RF_784, both of which correspond to *ccoN* genotype OTU_7 (Fig. 3.1), were enriched when bacteria from either lake were incubated with phytoplankton from TB and were both positively correlated with *Gymnodinium fuscum* in environmental time series observations (Fig. 3.4b). No indirect interactions were inferred because no bacterial ARISA fragments were correlated with either of these T-RFs and *G. fuscum*. In contrast, there was evidence that bacterial ARISA fragments 584 and 606 may mediate the negative effects of *Asterionella* and *Dinobryon*, respectively on *Polynucleobacter* T-RF RR_434 (Fig. 3.4c), which corresponds to OTU 30 and OTU 34 (Fig. 3.1). Seasonal abundance of *Polynucleobacter* T-RF RR_434 was negatively correlated with both *Asterionella* and *Dinobryon*, and positively correlated with ARISA fragments 584 and 606, which were each negatively correlated with one of the phytoplankton taxa (Fig. 3.4c). In the experimental phytoplankton treatments, RR_434, ARISA 584 and ARISA 606 exhibited

similarly significant decreases in abundance while not decreasing in no-phytoplankton control treatments (Fig. B.4), suggesting that other bacteria potentially mediate negative responses to phytoplankton.

DISCUSSION

Effect of phytoplankton on Polynucleobacter *subtype composition*

These results demonstrate that the *Polynucleobacer* composition within lake epilimnia changes over the course of phytoplankton seasonal succession due, in part, to subtype-specific responses to phytoplankton populations. Correlative environmental evidence for interaction between phytoplankton and *Polynucleobacter* subtypes is as strong as the evidence for interaction between phytoplankton and the bacterial community in these lakes (Kent et al., 2007). Repeatable change in the composition of *Polynucleobacter* subtypes in response to experimental manipulation of phytoplankton assemblages demonstrated that *Polynucleobacter ccoN* alleles differentiate ecologically distinct populations and confirmed the effect of phytoplankton on *Polynucleobacter* subtypes.

The importance of interactions between phytoplankton and *Polynucleobacter* in these lakes had previously been hypothesized following the observation that 48% of bacterial *glcD* gene sequences, which indicate genetic potential to use the algal exudate glycolate, had a high degree of similarity to the *glcD* gene from *Polynucleobacter necessarius* (Paver and Kent, 2010). Wu and Hahn (2006) proposed that PnecB *Polynucleobacter* depend on autochthonous (within-lake) substrates, which include algal-derived compounds, based on i) their occurrence in productive lakes, ii) their depth distribution mirroring chlorophyll *a* concentration, and iii) the large number of PnecB1 sequences generated from a phytoplankton-influenced experimental

system by Horner-Devine et al. (2003). Accordingly, PnecB *Polynucleobacter* were also detected in cultures of different phytoplankton species, with the highest abundances (5-16% of bacterial cells) observed in cryptophyte cultures (Šimek et al., 2011). In the same study, PnecC made up about 5% of bacterial cells in one culture of *Cryptomonas*, but were notably at very low abundance or not detected in the other nine cultures studied (Šimek et al., 2011). Given that over 85% of *Polynucleobacter* sequences in our humic lake study sites are consistently identified to the PnecC subgroup (Paver et al., 2013b; Youngblut et al., 2013), our results suggest that phytoplankton and specific subtypes of PnecC *Polynucleobacter* interact.

Our observations that phytoplankton differentially affect *Polynucleobacter* subtypes has important implications for understanding interactions between phytoplankton and bacterial communities. There is compelling evidence that phytoplankton blooms and seasonal succession affect bacterial community composition in a range of study systems, including the lakes investigated in this study (Arrieta and Herndl, 2002; Teeling et al., 2012; Paver et al., 2013b). Phytoplankton exudates have garnered support as a key mechanism for phytoplankton to affect bacteria (Sadro et al., 2011; Sarmento and Gasol, 2012; Nelson et al., 2013). To assess the degree of bacterial selectivity with respect to algal exudates, Sarmento and Gasol (2012) determined the percent of different types of marine bacteria actively taking up radiolabelled carbon compounds from different species of phytoplankton and a leucine control. The percent of cells taking up radiolabelled compounds varied widely depending on the phytoplankton species. For example, 75% of bacteria in the *Roseobacter* genus actively took up the leucine control while the percent taking up phytoplankton-derived radiolabelled compounds depended on the phytoplankton species: *Skeletonema* (10%), *Chaetoceros* (20%), *Synechococcus* (35%), and *Prochlorococcus* (0%) (Sarmento and Gasol, 2012). Within-taxon differentiation in substrate use like that

observed for 2-4 isolates within PnecC, PnecB, and PnecD *Polynucleobacter* subgroups (Jezbera et al., 2012) and 13 *Brevundimonas alba* isolates (Jaspers and Overmann, 2004) may explain both the variation observed in the percentage of cells of a particular genus actively taking up compounds from different phytoplankton species and variation in the response of *Polynucleobacter ccoN* subtypes to phytoplankton.

Experimental results from this study provide additional insight into how phytoplankton interact with *Polynucleobacter* subtypes. Initial differences in *Polynucleobacter* composition between phytoplankton and control treatments suggest that some *Polynucleobacter* live in close association with phytoplankton, either as endophytes living within cells or epiphytes living attached to cell surfaces. *P. necessarius* ssp. *necessarius* is an endosymbiont of a ciliate host assumed to be absent from the water column because the host has a benthic lifestyle (Heckmann and Schmidt, 1987; Vannini et al., 2007). In contrast, *P. necessarius* ssp. *asymbioticus* contains obligate free-living strains (Hahn et al., 2009; Jezberová et al., 2010). If the observed phytoplankton-associated *Polynucleobacter* subtypes belong to one of the described *P. necessarius* subspecies (PnecC), it suggests that either *P. necessarius* ssp. *necessarius* includes organisms that inhabit additional host species or *P. necessarius* ssp. *asymbioticus* contains host-associated organisms. Alternatively, phytoplankton-associated *Polynucleobacter* subtypes may belong to *P. rarus*, a species about which little ecological insight is available (Hahn et al., 2011) that accounts for ~5% of *Polynucleobacter* 16S rRNA sequences in this system (Shade et al., 2012; Paver et al., 2013b). In addition to phytoplankton-association, our data suggest that negative phytoplankton effects may be mediated through interaction with other bacteria (Fig. 3.4a). Based on these data, it is not possible to determine whether the effects of phytoplankton on the *Polynucleobacter* subtype are mediated by the bacterial taxon, the effects of phytoplankton

on the bacterial taxon are mediated by the *Polynucleobacter* subtype, or whether phytoplankton affect both the *Polynucleobacter* subtype and bacterial taxon individually.

Combined effects of environment and phytoplankton on Polynucleobacter *subtype composition*

Phytoplankton clearly influence the composition of *Polynucleobacter* subtypes; however, the effects of phytoplankton act in concert with other factors. In environmental time series observations, lake (CB, SSB, or TB) and the year of sample collection (2003 or 2008) affected the composition of *Polynucleobacter* (Fig. B.2). Lake- and year-specific *Polynucleobacter* composition may help explain why no correlations between pairs of phytoplankton and *Polynucleobacter* T-RFs were observed across different lakes or across different years in the same lake. Multiple seasonally variable factors may also affect *Polynucleobacter* and interfere with the ability to detect the influence of phytoplankton. For example, an intense period of early-summer grazing that has been observed over multiple years in CB has been demonstrated to affect bacterial community composition as well as the percent of filamentous bacteria (Kent et al., 2004; Kent et al., 2006).

Each lake had a characteristic *Polynucleobacter* assemblage that was maintained across sampling years (Fig. 3.1, Fig. B.2). For example, OTU_31 was consistently detected in TB clone libraries but was not found in clone libraries constructed from any other humic lake surveyed (Fig. 3.1; Fig. B.5)(Youngblut et al., 2013). The effect of lake may be due to differences in specific biotic and physico-chemical characteristics of each lake. Some phytoplankton species are shared among lakes, but each lake tends to have a characteristic dominant phytoplankton assemblage (Paver et al., 2013b). Additionally, TB tends to have lower pH and higher concentrations of total nitrogen, total phosphorus, and dissolved organic carbon than CB and

SSB (Paver et al., 2013b). Observations of CB and TB collected over the past decade by the North Temperate Lake Long Term Ecological Research (NTL-LTER) project confirm that pH is typically slightly higher in CB (CB: average 5.2, range 4.7-5.7, TB: average 4.9, range 4.5-5.6) and dissolved organic carbon is typically higher in TB (CB: average 11.0 mg/l, range 5.5-20.0 mg/l; TB: average 19.5 mg/l, range 11.0-30.2 mg/l) (http://lter.limnology.wisc.edu). Jezbera et al. (2011) previously observed pH / conductivity, altitude / surface area, dissolved organic carbon and oxygen concentration preferences among 13 *P. n. asymbioticus* (PnecC) subgroups, which resulted in complete habitat separation between certain subgroups (Jezbera et al., 2011). However, the range of physico-chemical parameters observed across the 121 freshwater habitats surveyed by Jezbera and colleages (e.g., pH 4.4-8.5) was much greater than the variation observed in our suite of humic lakes.

Observation of year-specific differences in *Polynucleobacter* subtype composition across samples collected from each lake (Fig. B.2) indicates that regional-scale factors affect *Polynucleobacter* composition. One factor may be the regional species pool. For example, the phytoplankton *Trachelomonas* was observed briefly (2-3 weeks) in all three lakes in 2008, but not detected in 2003. Seasonal temperatures affect the timing and duration of the ice-free period for north temperate lakes, which is the time period where microbial communities are most variable, and may also contribute to year-specific differences (Kent et al., 2004). Ice-off occurred about 9 days earlier in 2003 compared to 2008 (http://lter.limnology.wisc.edu). High precipitation levels prior to 2003 (2001: 640mm; 2002: 710mm) and low precipitation levels prior to 2008 (2006: 384mm; 2007: 401mm) could also have contributed to year-specific differences in *Polynucleobacter* composition (http://lter.limnology.wisc.edu). Timing of ice-out

and spring and summer precipitation have been observed to affect dissolved organic carbon dynamics in lakes (Pace and Cole, 2002).

At a seasonal time-scale, changes in environmental factors within a lake also affect *Polynucleobacter* subtype composition. Changes in the lake environment from May through August (e.g., temperature, nutrient availability, dissolved organic carbon) and the interaction between phytoplankton and environment also explained a substantial amount of the temporal change in the composition of *Polynucleobacter* subtypes. Differences in seasonal dynamics have previously been observed for *Polynucleobacter* subgroups PnecC, PnecB, and PnecD (Wu and Hahn, 2006a). Conductivity and pH explained observed patterns in a deep oligo-mesotrophic lake while temperature explained observed patterns in a shallow eutrophic-hypertrophic lake (Wu and Hahn, 2006a). Importance of interaction between the environment and phytoplankton is supported by the observation that water temperature and biomass of chrysophytes and dinoflagellates were better at predicting annual abundance cycles of PnecB in Lake Mondsee than temperature alone (Wu and Hahn, 2006b).

Limitations

There are certain methodological limitations of our study design that merit discussion. First, *Polynucleobacter* subtype composition was characterized using relative abundance-based methods, which may lead to misinterpretation of terminal restriction fragment (T-RF) responses. For example, it is possible for a T-RF subtype to decrease in relative abundance while that subtype either maintains a consistent population size or actually increases in abundance if the abundances of other subtypes increase more rapidly. Additionally, some *Polynucleobacter ccoN* genotypes could not be differentiated by T-RFLP (Fig. 3.1). Despite the potential for false

negative results due to misclassification of subtypes with weaker responses to phytoplankton and the aggregation of certain subtypes, our characterization approach is useful for identifying the most responsive subtypes and confirming that phytoplankton affect the composition of *Polynucleobacter* subtypes. Second, phytoplankton collection using differential filtration enabled the use of native phytoplankton assemblages, but also introduced potential confounding effects of including additional organisms in phytoplankton treatments (Paver et al., 2013b).

Conclusion

We present evidence that *ccoN* genotypes of *Polynucleobacter* represent ecologically distinct populations that differ in their response to phytoplankton and other seasonal drivers. The composition of *Polynucleobacter* based on analysis of the *ccoN* gene is temporally variable and observed genetic differentiation between *ccoN* alleles from selected sampling dates suggests that seasonal patterns in *Polynucleobacter* composition result from selection for divergent lineages. Observed effects of phytoplankton are likely due to certain subtypes inhabiting phytoplankton cells and differentiation in substrate use among *Polynucleobacter* subtypes, similar to what has been observed for isolates within the freshwater bacterial species *Brevundimonas alba* (Jaspers and Overmann, 2004). Additionally, phytoplankton effects on bacteria operate in combination with other factors to influence temporal patterns. Species interactions, including those with phytoplankton, differentiate among closely related populations and are important for understanding and predicting temporal and spatial changes in bacterial community composition.

EXPERIMENTAL PROCEDURES

Study sites and sample collection

Crystal Bog (CB; 46°00'26.8"N, 89°36'22.5"W), South Sparkling Bog (SSB; 46°00'13.6"N 89°42'19.9"W), and Trout Bog (TB; 46°02'27.5"N, 89°41'09.6"W) are humic lakes located in the Northern Highland Lake District of Northern Wisconsin. These lakes have high levels of DOC (>9.5 mg/l), are darkly stained, and are characterized by acidic pH. Of the three lakes, TB typically has higher total nitrogen, total phosphorus, and dissolved organic carbon concentrations than CB and SSB (Paver et al., 2013b). The dominant phytoplankton taxa in terms of biomass in TB (*Gymnodinium fuscum*, *Asterionella*, *Cryptomonas*) also tend to differ from the dominant phytoplankton in CB (*Synura*, *P. umbonatum*, *P. limbatum*) and SSB (*P. limbatum*, *Synura*, *G. fuscum*) (Paver et al., 2013b). Integrated epilimnion samples were collected weekly from each lake between May and August in 2003 and 2008 as described by Kent et al. (2007). Microorganisms were collected onto 0.22µm filters (Supor-200; Pall Gelman, East Hills, NY, USA) and frozen at -20°C. Subsamples were preserved in 2% glutaraldehyde for identification and enumeration of phytoplankton using microscopy. Every other week total N, total P, and DOC were determined according to standard protocols of the North Temperate Lakes Long Term Ecological Research project (http://lter.limnology.wisc.edu/)(Kent et al., 2007).

Algal-exchange mesocosm experiment

Bacterial communities from SSB and TB were incubated with native algal assemblages from SSB, TB, or filter-sterilized SSB water as a no-phytoplankton control in a full factorial design with three replicates of each treatment as described by Paver et al. (Paver et al., 2013b). An integrated epilimnion sampler was used to collect water from the first two meters of each

lake. A subset of the lake water was filtered through a 1µm Polycap AS cartridge filter (Whatman, Piscataway, NJ, USA) to separate bacterial communities from larger organisms. Native algal assemblages were collected by filtering lake water through a 100µm nylon mesh (Spectrum Laboratories, Rancho Dominguez, CA, USA) to remove zooplankton and collecting, then rinsing algal cells captured on a 20µm nylon mesh (Spectrum Laboratories), which allowed smaller organisms such as heterotrophic nanoflagellates and bacteria to pass through. Phytoplankton collected on the 20µm mesh were resuspended in 0.2µm filter-sterilized SSB water, concentrating phytoplankton from 50L of lake water to 3L of sterilized water. All combinations of bacteria (5L of 1µm filtered water) from each lake and 0.3L of concentrated phytoplankton from each lake or a no-phytoplankton control (0.3L of 0.2µm filter-sterilized and autoclaved CB water) were combined in 10L LDPE cubitainers (I-Chem, Rockwood, TN, USA) and then added to 250mL clear glass bottles (Wheaton Scientific, Millville, NJ, USA). Bottles were destructively sampled before and after a 5-day incubation. Microorganisms from 100mL subsamples of each mesocosm were collected onto 0.22µm filters (Supor-200; Pall Gelman, East Hills, NY, USA) and frozen at -20°C.

ccoN *gene sequencing*

Polynucleobacter-specific primers Pnuc0453F (5'-CAGYCAATTTGCCATCGTTAC-3') and Pnuc0453R (5'-GTCATGATGCCGTTGATC-3') were used to amplify the protein-encoding gene cytochrome C oxidase, cbb3-type, subunit I (*ccoN*) of bacteria within the *Polynucleobacter* genus in select time series samples (Youngblut et al., 2013). We followed the PCR amplification conditions and cloning protocol described by Youngblut and colleagues

(2013). Sanger sequencing was conducted from both ends of the vector insert at the Keck Center for Comparative and Functional Genomics at the University of Illinois.

Sequence and phylogenetic analysis

Sequences were assembled into contigs and manually screened for miscalled bases using Sequencher v 4.7 (Gene Codes Corporation, Ann Arbor, MI, USA). Assembled sequences were manually aligned with MacClade version 4.08 (Maddison and Maddison, 2000). Sequences were then grouped into operational taxonomic units at the >99.5% similarity level and rarefaction curves were generated using mothur v 1.15.1. (Schloss, 2009). Clone libraries were subsampled so that each library contained the same number of sequences using mothur. The fixation index (F_{ST}) was calculated for each pair of clone libraries using Arlequin v 3.5 (Weir and Cockerham, 1984; Excoffier and L. Lischer, 2010). Reference sequences were added to an alignment with one representative from each OTU, which was modified using the L-INS-i option in online MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/). RAxML Black Box v 7.2.8 was then used to create a maximum likelihood tree from the nucleotide multiple sequence alignment using the Gamma model of rate heterogeneity (Stamatakis et al., 2008).

Terminal restriction fragment length polymorphism

Forward and reverse terminal restriction fragment sizes were predicted for *Polynucleobacter ccoN* OTUs digested with each restriction enzyme available from New England Biolabs compatible with buffer 4 using TRiFLE (http://cegg.unige.ch/trifle/trifle.jnlp) (Junier et al., 2008). *HhaI* and *RsaI* were determined to be the enzyme combination that best differentiated among *ccoN* gene sequences. Pnuc0453F and Pnuc0453R were labeled at the 5'

end with phosphoramidite dyes HEX and 6-FAM, respectively. Polymerase chain reactions contained 0.8 mM deoxynucleoside triphosphates (Promega, Madison, WI, USA), 0.2 μM of each primer, 1x PCR buffer with BSA (Cat# 1772; Idaho Technology Inc., Salt Lake City, Utah, USA), 2.4 mM final concentration of $MgCl_2$ and 0.05 U μl-1 GoTaq (Promega). PCR cycles consisted of a 2 min initial denaturation at 94°C, followed by 26 cycles of 94°C for 60s, 58°C for 90s, and 72°C for 90s and a final extension of 72°C for 5 min (MasterCycler Gradient, Eppendorf AG, Hamburg Germany). MinElute PCR purification (Qiagen, Valencia, CA, USA) was used to concentrate three 50μl reactions and remove excess primers, nucleotides, and salts (Qiagen, Valencia, CA, USA). Purified PCR products were digested in single-enzyme incubations containing *HhaI* and *RsaI* (New England BioLabs Inc., Ipswich, MA, USA). Digested PCR products combined with the ABI GeneScan ROX 1000 size standard were analyzed by denaturing capillary electrophoresis using the ABI GeneScan ROX 1000 size standard and ABI 3730XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the W. M. Keck Center for Functional Genomics at the University of Illinois. GeneMarker version 1.75 software (SoftGenetics, State College, PA, USA) was used to carry out size-calling, profile alignment, and automatic grouping of terminal restriction fragments (T-RFs). A peak threshold of 50 fluorescence units was used to exclude background fluoresce while including the maximum number of peaks. To account for run-to-run variation, the area under each peak (signal strength) was normalized to the total area of all peaks in each electropherogram. Normalized data from each enzyme was concatenated for each sample (Fierer et al., 2003; Paver and Kent, 2010; Peralta et al., 2010).

Statistical analysis

Pairwise Bray-Curtis similarities of environmental time series *Polynucleobacter* assemblages characterized using T-RFLP were visualized using non-metric multidimensional scaling (MDS) ordination using PRIMER version 6 (PRIMER-E Ltd, Plymouth Marine Laboratory, UK)(Clarke and Warwick, 2001). The temporal variation in *Polynucleobacter* composition explained by phytoplankton community composition, environmental factors, and the interaction between phytoplankton and the environment within each time series was determined using partial canonical correspondence analysis in Canoco 4.5.1 (Biometris-Plant Research International, Wageningen, The Netherlands) (ter Braak and Smilauer, 2002). Local similarity analysis (LSA) and Pearson correlations were used to detect *Polynucleobacter* T-RFs and phytoplankton populations exhibiting correlated patterns of abundance in each time series (e.g. SSB 2003)(Ruan et al., 2006). LSA, Pearson correlations and calculation of corresponding p- and q-values (calculated to adjust for multiple comparisons) were performed using the LSA code developed by Ruan and colleagues (2006) in the R statistical environment (R Development Core Team, 2010). Significance of local similarity scores was based on 1000 permutations and no lag. Phytoplankton-*Polynucleobacter* pairs with local similarity scores greater than |0.3| and p-values <0.001 were considered significantly correlated (Shade et al., 2010). LSA networks were visualized using Cytoscape v. 2.6.1 (Shannon et al., 2003).

MDS ordination was additionally used to visualize pairwise Bray-Curtis similarities of mesocosm samples before and after incubation using PRIMER version 6 (Clarke and Warwick, 2001). PERMANOVA, a non-parametric multivariate analysis of variance that generates p-values using permutations, was used to test the effect of bacterial source (SSB, TB) and phytoplankton treatment (SSB, TB, none) on mesocosm *Polynucleobacter* composition before

and after incubation (Anderson, 2001; McArdle and Anderson, 2001). PERMANOVA tests were run using the adonis function from the vegan package (Oksanen et al., 2011) in the R statistical environment (R Development Core Team, 2010). The SIMPER analysis in PRIMER version 6 was used to determine *Polynucleobacter* T-RFs differentiating each phytoplankton treatment from the corresponding no-phytoplankton control before and after incubation (PRIMER-E Ltd, Plymouth Marine Laboratory, UK) (Clarke and Warwick, 2001). Time course differential analysis in the Extraction of Differential Gene Expression (EDGE) software (version 1.1.291) was used to identify bacterial taxa displaying differential responses in phytoplankton treatments compared to the corresponding no-phytoplankton control (Storey et al., 2005; Leek et al., 2006). Originally designed to identify differentially expressed genes, analyses in the EDGE package have previously been used to detect bacterial operational taxonomic units with differential responses to incubation in epilimnion and hypolimnion lake layers (Shade et al., 2010) and bacterial response to phytoplankton treatments (Paver et al., 2013b).

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TABLES

Table 3.1. *Polynucleobacter* population differentiation among clone libraries determined by fixation index calculations (F_{ST}). Significant F_{ST} values ($p<0.0001$) are indicated by bold font, dark grey shading indicates calculations comparing populations collected from the same lake in the same year and light grey shading indicates populations collected from the same lake in different years.

	CB 14Jul03	SSB 03Jun03	SSB 08July03	SSB 19Aug03	SSB 01May08	SSB 03Jul08	TB 24Jun03	TB 30Apr08	TB 04Jul08
CB									
14Jul03	0								
SSB									
03Jun03	**0.100**	0							
08July03	**0.189**	**0.078**	0						
19Aug03	**0.225**	**0.160**	0.045	0					
01May08	**0.342**	**0.197**	**0.344**	**0.442**	0				
03Jul08	**0.195**	**0.122**	0.010	0.020	**0.383**	0			
TB									
24Jun03	**0.220**	**0.111**	**0.022**	**0.058**	**0.330**	0.024	0		
30Apr08	**0.240**	**0.136**	**0.240**	**0.331**	**0.106**	**0.273**	**0.217**	0	
04Jul08	**0.193**	**0.083**	**0.156**	**0.236**	**0.100**	**0.182**	**0.132**	0.006	0

Table 3.2. Results from PERMANOVA testing the effect of bacteria and phytoplankton source lake on bacterial community composition before (day 0) and after (day 5) incubation.

		df	Sum of Squares	F	P	R^2
Day 0						
	Bacteria	1	0.13	8.4	0.006	0.27
	Phytoplankton	2	0.11	3.8	0.027	0.24
	Bacteria*Phytoplankton	2	0.05	1.5	0.211	0.10
	Residuals	12	0.18			0.38
Day 5						
	Bacteria	1	0.29	13.6	<0.001	0.23
	Phytoplankton	2	0.30	13.8	<0.001	0.46
	Bacteria*Phytoplankton	2	0.06	2.8	0.013	0.06
	Residuals	12	0.02			0.20

Table 3.3. Percent of the difference between phytoplankton and the corresponding no-phytoplankton control treatments explained by *Polynucleobacter* OTUs that were enriched or depleted in phytoplankton treatments following incubation and those that were consistently enriched or depleted before and after incubation determined by SIMPER analysis.

	SSB Phytoplankton		TB Phytoplankton		
Bacterial source:	SSB	TB	SSB	TB	Average
Phytoplankton-enriched following incubation	16.3	13.4	24.8	16.0	17.6
Consistently phytoplankton-enriched	25.1	34.3	28.0	28.9	29.0
Phytoplankton-depleted following incubation	20.8	7.0	11.5	24.8	16.0
Consistently phytoplankton-depleted	36.2	43.6	34.3	26.0	35.0

FIGURES

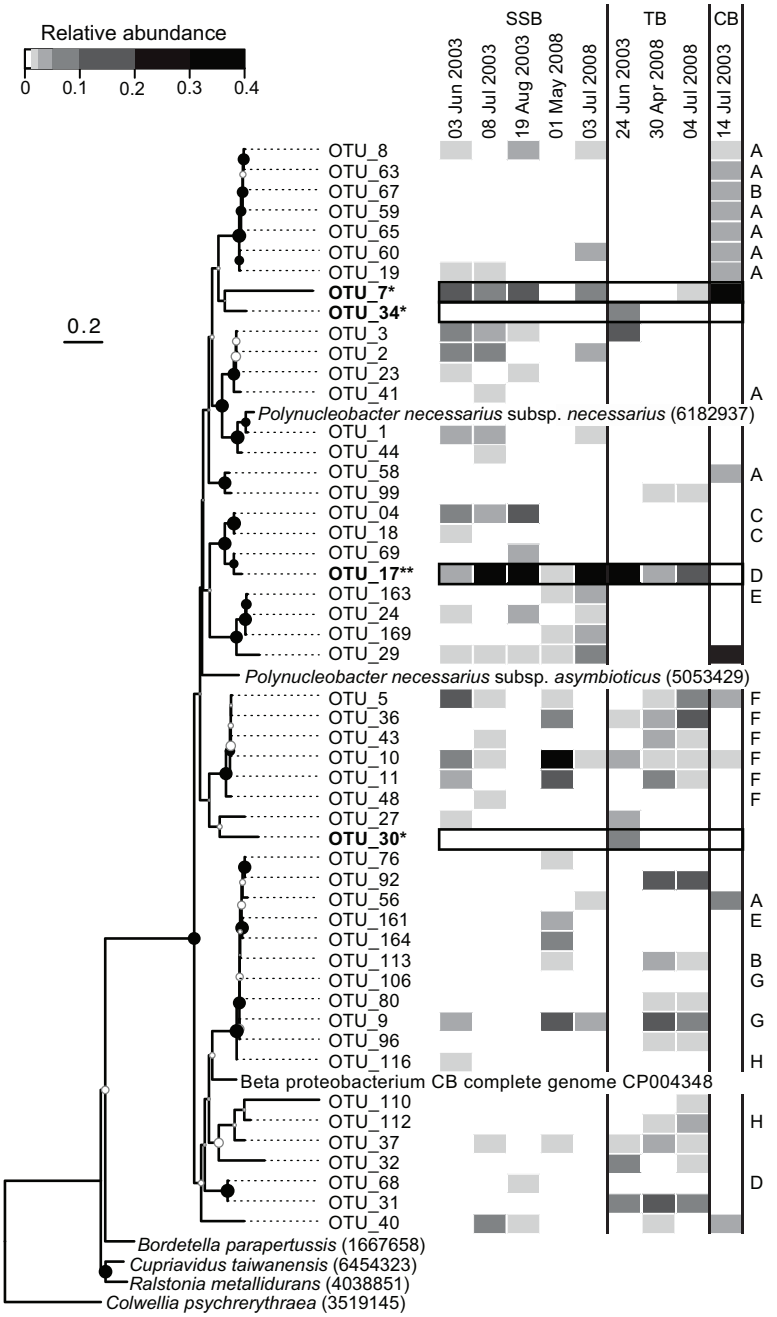

Figure 3.1. Maximum likelihood tree of *Polynucleobacter* cyctochrome c oxidase I (*ccoN*) gene OTUs from the current study determined by mothur at the >99.5% similarity level and select sequences from GenBank with gene IDs in parentheses. Based on 16S rRNA analysis, 'Beta proteobacterium CB' is affiliated with the PnecC tribe of *Polynucleobacter*, as are both *P. necessarius* sequences. The heat map indicates the relative abundance of *Polynucleobacter* OTUs in each clone library. Letters (A-H) indicate groups of OTUs that yield identical terminal restriction fragment lengths. Scale bar indicates 0.2 changes per nucleotide. Node size corresponds to bootstrap value; values <70 are indicated by open circles. Asterisk (*) denotes OTUs included in Figure 3.4. Double asterisk (**) denotes an epilimnion-specialist OTU described by Youngblut and colleagues (2013).

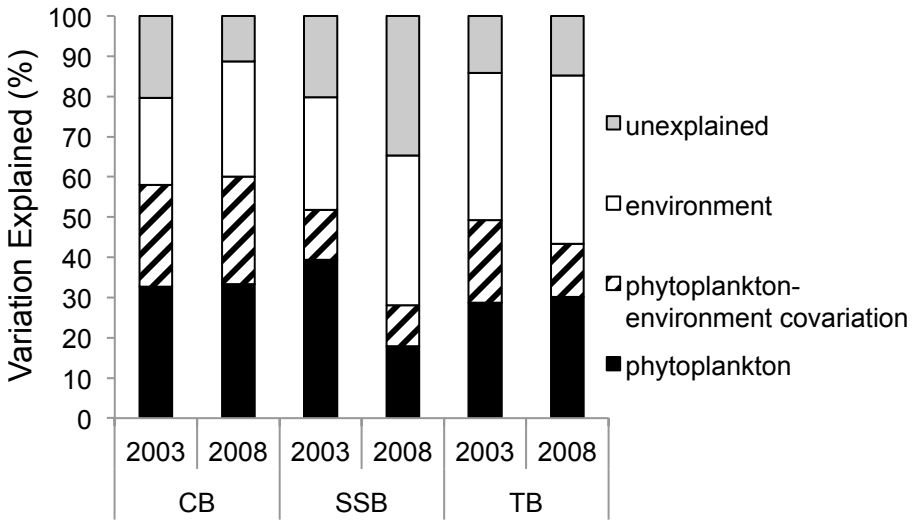

Figure 3.2. Variation in the composition of *Polynucleobacter* in samples collected weekly from May through August over two years from three humic lakes explained by changes in the abundance of phytoplankton populations, covariation between phytoplankton and the environment, environmental factors or unexplained as determined using partial canonical correspondence analysis.

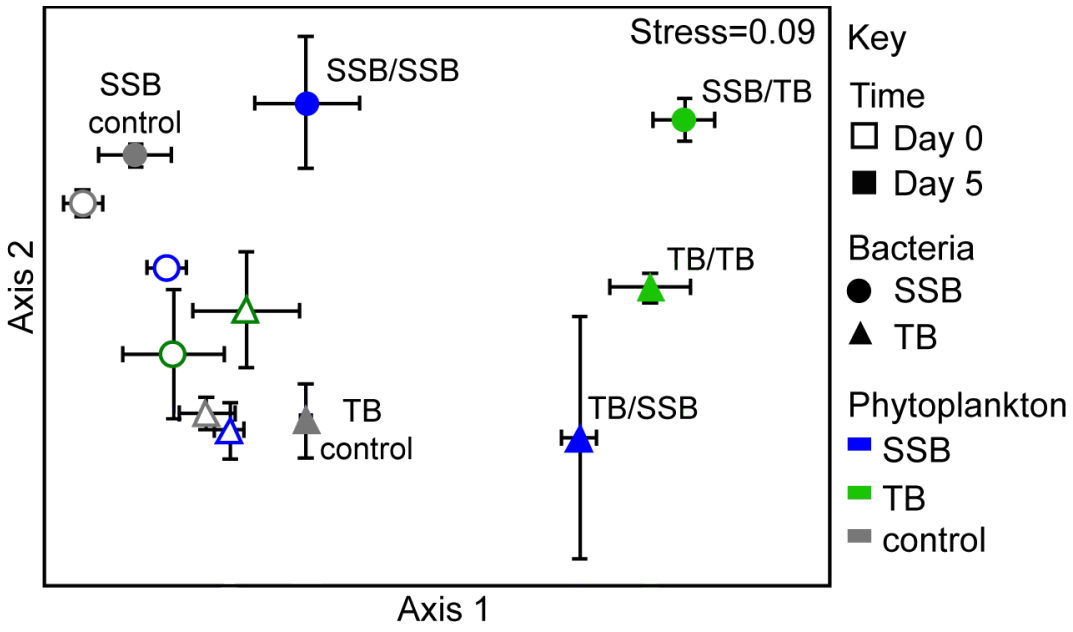

Figure 3.3. Non-metric multi-dimensional scaling ordination of pairwise Bray-Curtis similarity comparing *Polynucleobacter* assemblages characterized using *ccoN* gene T-RFLP in mesocosms containing bacteria from SSB and TB combined with phytoplankton from SSB, TB, or no phytoplankton (control) before and after a five-day incubation.

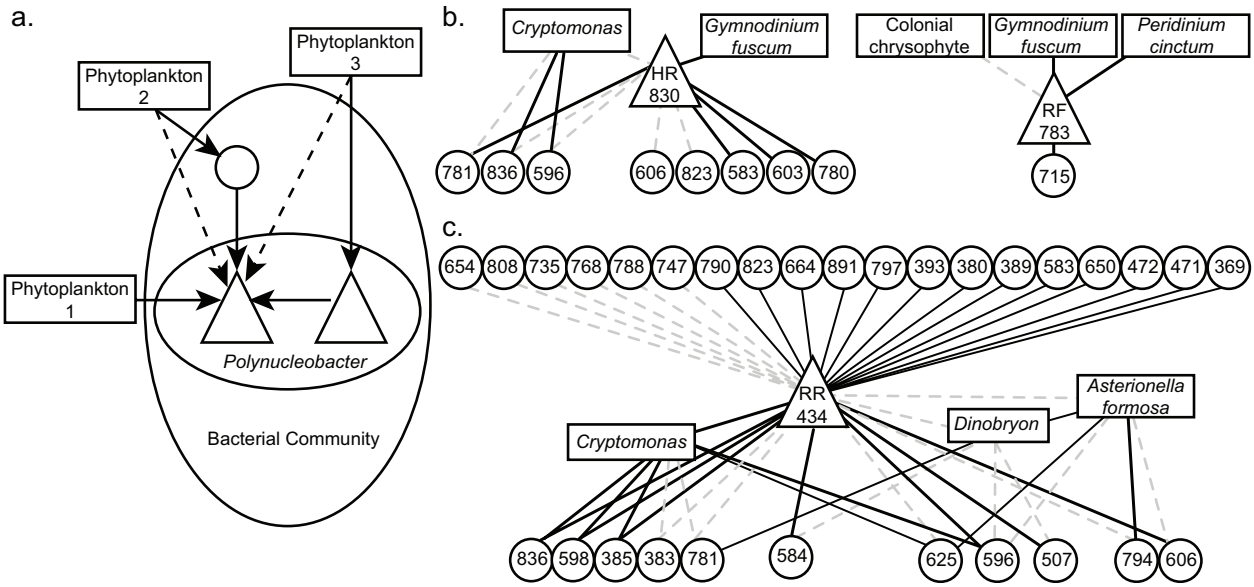

Figure 3.4. Phytoplankton can affect *Polynucleobacter* subtypes (1) directly, (2) mediated by interaction with other bacteria, or (3) mediated by interaction with another *Polynucleobacter* subtype (a). To detect possible indirect interactions, positive (solid lines) and negative (dotted lines) correlations between the relative abundance of phytoplankton-responsive *Polynucleobacter* T-RFs (triangles), phytoplankton (rectangles) and bacteria (circles) in environmental time series samples were determined using local similarity analysis. *Polynucleobacter*-responsive T-RFs shown correspond to *ccoN* genotype OTU_7 (b) and ccoN genotypes OTU_30 and OTU_34 (c).

CHAPTER 4

PHYTOPLANKTON AFFECT CHANGES IN LAKE BACTERIAL COMMUNITY COMPOSITION IN RESPONSE TO LIGHT AND TEMPERATURE

ABSTRACT

Interactions with phytoplankton influence bacterial community composition and temporal patterns. Environmental conditions, including light and temperature, can alter interactions between phytoplankton and bacteria and also influence bacterial communities directly. Our objective was to determine how phytoplankton, temperature, and light act in concert to affect bacterial community composition. In a full factorial design, bacteria from two humic lakes were combined with phytoplankton assemblages from each lake ("home" or "away") or a no-phytoplankton control and incubated for 5 days under all combinations of light (surface, ~25% surface irradiance) and temperature (5 levels from 10°C to 25°C). Light availability directly affected bacterial community composition, potentially due to stimulating the growth of phototrophic bacteria. Temperature effects were largely phytoplankton-mediated and explained the most bacterial community variation in "away" phytoplankton treatments. We hypothesize that the enhanced effect of "away" phytoplankton on bacteria is due to the availability of a different pool of exudates than bacteria had become acclimated to in their "home" lake. The response of certain bacterial operational taxonomic units to phytoplankton treatments depended on light and temperature including ones identified to the betI-A (*Limnohabitans*) and Pnec clades and Chlamydiales order. Further investigation into the mechanisms of phytoplankton influence and a trait-based understanding of bacterial taxa will be instrumental in developing a predictive framework for bacterial community temporal patterns.

INTRODUCTION

Species interactions, environmental conditions, and stochastic processes affect the abundance, diversity, and distribution of organisms in the environment. However, the relative importance of these factors and how they interact to determine community composition remain poorly understood (Agrawal et al., 2007). Environmental conditions have been shown to affect the importance as well as the outcome of species interactions (Bronstein, 1994; Chase, 2010; He et al., 2013). To predict how communities change over time, it is therefore necessary to identify species interactions affecting community composition and elucidate how these interactions change under different environmental contexts. A framework for explaining community dynamics will provide a backdrop for examining mechanisms that create and maintain diversity and enhance our ability to forecast community responses to environmental change.

Lake bacterioplankton are an ideal system to examine the effects of multiple interacting factors on community composition due to annually repeating patterns of community assembly (Shade et al., 2007; Nelson, 2009; Kara et al., 2013) and the relative ease of experimental manipulation (Kent et al., 2006; Adams et al., 2010; Paver and Kent, 2010). Phytoplankton seasonal succession contributes to observed temporal patterns in lake bacterial community composition through interactions between specific phytoplankton and bacterial populations (Kent et al., 2007; Paver et al., 2013). Light and temperature availability vary temporally and with depth and can affect bacterial community composition directly as well as by altering the interactions between phytoplankton and bacteria (Zlotnik and Dubinsky, 1989; Parker and Armbrust, 2005; Zubkov, 2009; Hortnagl et al., 2011).

Light availability can enhance or inhibit the growth of different bacterial populations. Rhodopsin-containing bacteria and aerobic anoxygenic phototrophs can use light energy to

supplement their energy requirements (Zubkov, 2009). Genes for photoheterotrophy were found in 10-23% of single-amplified genomes from three temperate freshwater lakes (Martinez-Garcia et al., 2012). Additionally, the ribulose 1,5-bisphosphate carboxylase/oxygenase gene (RuBisCo) has been detected in non-cyanobacterial freshwater bacteria, suggesting that light may also affect chemoautotrophic bacteria (Martinez-Garcia et al., 2012). Ultraviolet (UV) radiation can also affect bacterial community composition. UV radiation affects bacterial growth efficiency and has a strain-specific effect on bacterial metabolic rates (Hortnagl et al., 2011). Additionally, UV radiation can transform organic matter, resulting in high concentrations of low molecular weight dissolved organic carbon and oxidative stress, which can select for specific members of the bacterial community adapted to these conditions (Paul et al., 2012).

Temperature can affect both bacterial community composition and rates of bacterial processes. In general, a temperature increase of 10 °C within an enzyme's tolerance range results in an increase in enzyme activity by a factor of 2 (Q_{10} value; range: 1.5-4)(Overmann, 2013). As a result, there is a positive relationship between bacterial growth rate and temperature in freshwater and marine habitats (White et al., 1991). Bacteria have diverse optimal growth temperatures and temperature ranges, such that temperature can determine outcomes of competition between different bacterial populations (Upton et al., 1990). Incubation of arctic lake bacteria at temperatures ranging from 8°C to 20°C affects bacterial community composition as well as bacterial production (Adams et al., 2010). Despite the generally positive relationship between enzyme activity and temperature, Adams and colleagues (2010) observed temperature optima at 12°C and 20°C, suggesting that the effect of temperature is complex and potentially dependent on bacterial community composition.

In addition to direct effects, light and temperature can influence bacterial community composition through modifying their interactions with phytoplankton. Light and temperature have the potential to induce changes in phytoplankton community composition (Litchman and Klausmeier, 2008), which has been shown to affect the composition of bacterial communities (Kent et al., 2007; Paver et al., 2013). Light and temperature can also affect bacteria through inducing changes in algal exudate release. Bacteria rapidly use labile exudates (e.g., sugars, amino acids) released by phytoplankton (Larsson and Hagström, 1979; Sadro et al., 2011), and exudate composition influences bacterial community structure (Sarmento and Gasol, 2012; Nelson et al., 2013). Phytoplankton release labile exudates that vary in quantity and composition depending on factors including phytoplankton species (Hellebust, 1965; Fogg, 1983), phytoplankton growth stage (Grossart et al., 2005), and environmental conditions (Zlotnik and Dubinsky, 1989; Parker and Armbrust, 2005; Panzenbock, 2007). Concentration of extracellular organic carbon excreted from different cultures of phytoplankton has been shown to increase with increasing temperature up to a species-specific temperature optimum and light levels (Zlotnik and Dubinsky, 1989). In a marine diatom, the release of glycolate, a common algal exudate and product of photorespiration, is elevated at high light levels, especially at warmer temperatures (Parker and Armbrust, 2005). Grazing of bacterial communities by mixotrophic phytoplankton that can carry out photosynthesis and ingest bacteria can also be influenced by light and temperature. As temperature increases, mixotrophic phytoplankton are theorized to become more heterotrophic; this has been experimentally demonstrated with the chrysophyte *Ochromonas* sp. (Wilken et al., 2013). Both positive and negative correlations between light availability and heterotrophic grazing on bacteria have been observed (Caron et al., 1993; Jones et al., 1993; Hansen and Nielsen, 1997; Holen, 1999; Skovgaard et al., 2000).

Our objective was to characterize the direct and interactive effects of phytoplankton, light, and temperature on bacterial community composition from two humic lakes in Northern Wisconsin where interaction with phytoplankton has been demonstrated to affect bacterial community dynamics (Kent et al., 2007; Paver et al., 2013). Change in light and temperature with depth is especially pronounced in darkly stained humic lakes where chromophoric dissolved organic matter absorbs light, especially short wavelengths in the ultraviolent range, and therefore heat (Huovinen et al., 2003). We specifically sought to determine whether the influence of light and temperature on bacterial communities is mediated by phytoplankton. If light and temperature affect bacteria through modifying interactions with phytoplankton, then it is expected that the variation in bacterial community composition due to light and temperature will be greater when phytoplankton are present than when they are absent.

MATERIALS AND METHODS

Study sites

South Sparkling Bog (SSB; 46°00'13.6"N, 89°42'19.9"W) and Trout Bog (TB; 46°02'27.5"N, 89°41'09.6"W) are two north temperate humic lakes in Vilas County, Wisconsin that have been studied as part of the North Temperate Lakes Microbial Observatory. SSB and TB were selected for their similarity in maximum depth (~8 m) and differences in phytoplankton community composition (Paver et al., 2013). Acidic pH and high levels of dissolved organic carbon characterize these lakes. TB typically has higher concentrations of total nitrogen and total phosphorus than SSB (Kent et al., 2007). Based on light attenuation data collected in 2010 (Read, unpublished data), 0.1% of 320 nm surface irradiance was available at 0.20 m in SSB and

0.19 m in TB and 0.1% of 380 nm surface irradiance was available at 0.5 m in SSB and 0.45 m in TB.

Experimental design

We conducted a multi-factorial microcosm experiment to determine the direct and interaction effects of phytoplankton presence and composition, temperature, and light on bacterial community composition. On 6 July 2011, microorganisms were collected from SSB and TB integrated epilimnion samples (0-1 m). Filtration through a 1 μm Polycap AS cartridge filter (Whatman, Piscataway, NJ, USA) was used to separate bacteria from larger organisms. Phytoplankton assemblages were collected by filtering lake water through a 100 μm nylon mesh (Spectrum Laboratories, Rancho Dominguez, CA, USA) to remove zooplankton and collecting, then rinsing phytoplankton cells captured on a 20 μm nylon mesh with SSB water filter-sterilized through a 0.2 μm Polycap AS cartridge filter (Spectrum Laboratories), which allowed smaller organisms such as heterotrophic nanoflagellates and bacteria to pass through. Phytoplankton collected on 20 μm mesh were resuspended in 0.2 μm filter-sterilized water from SSB, concentrating phytoplankton from 40 L of lake water to 2.5 L of sterilized water. All combinations of bacteria from each lake (5 L of 1 μm filtered water) were combined with 0.25 L of concentrated phytoplankton from one of the two lakes lake or a no-phytoplankton control (0.25 L of 0.2 μm filter-sterilized and SSB water) in triplicate 10 L LDPE cubitainers (I-Chem, Rockwood, TN, USA). Combined bacteria and phytoplankton were gently inverted to mix and then added to 500 ml clear glass bottles (Wheaton, Millville, NJ, USA) in a predetermined, random order (33 bottles/ treatment).

For each bacteria-phytoplankton combination, three bottles were used to characterize the initial community composition and three bottles were incubated for five days under each of five temperatures and two light levels (Fig. 4.1). Temperature treatments were established and maintained by continuously pumping defined proportions of high temperature (~25°C) surface water and low temperature (~5°C) subsurface water (1:0, 3:1, 1:1, 1:3, 0:1) into floating plastic container incubators (73 cm x 53 cm x 46 cm, The Container Store, Coppell, TX, USA) (Fig. C.1). High light and low light treatments were established by incubating bottles at the surface and bottom (~25% of surface irradiance) of each floating container incubator. Light and temperature conditions were monitored throughout the incubation using HOBO light and temperature pendant data loggers (Onset, Pocasset, MA, USA) with three loggers placed in each container: two at opposite corners at the surface and one on the bottom.

Microbial community analysis

Microorganisms from initial samples and from each bottle microcosm following incubation were concentrated onto 0.22 μm filters (Supor-200; Pall Gelman, East Hills, NY) and frozen at -20°C. DNA was extracted using FastDNA purification kits (MP Biomedicals, Solon, OH, USA). Bacterial community composition was characterized using automated ribosomal intergenic spacer analysis (ARISA), a community fingerprinting method based on length heterogeneity of the intergenic spacer region between bacterial 16S and 23S rRNA genes (Fisher and Triplett, 1999). Reagent concentrations and PCR cycling conditions were described in Chapter 2 (Paver et al., 2013). Fluorescently labeled ARISA PCR amplicons were combined with a custom 100 – 1250 bp Rhodamine X-labeled internal size standard (Bioventures, Murfreesboro, TN) and analyzed by the Keck Center for Functional Genomics at the University

of Illinois via denaturing capillary electrophoresis using an ABI 3730XL Genetic Analyzer (Applied Biosystems Inc., Carlsbad, California, USA). Electropherograms from each sample were aligned and peaks greater than 500 fluorescence units were sized and grouped into bins of operational taxonomic units using GeneMarker version 1.95 (SoftGenetics, State College, PA, USA). ARISA fragments known to correspond to chloroplasts were removed from the analysis. The signal strength of each peak was normalized to account for run-to-run variations in signal detection by dividing the area of individual peaks by the total fluorescence (area) detected in each profile.

Bacteria and phytoplankton from initial samples and microcosms incubated with the warmest and coldest temperatures at both light levels were characterized using 454 tag-pyrosequencing of the 16S rRNA and 23S rRNA genes, respectively. Primer sequences were designed to produce one-way reads using the emPCR Lib-L chemistry (Roche Applied Science, Basel, Switzerland). Each 25 μL PCR reaction contained 0.02 Units/ μL Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, MA, USA), 1 nM Phusion HF buffer, 0.2 mM of each dNTP (New England Biolabs, Ipswich, MA, USA), and 0.2 μM of each primer. For 16S rRNA gene sequencing, the V3-V5 region was amplified using barcoded primer A with template specific primer 806 R (earth microbiome project) and primer B with template specific primer Bakt_341F (Herlemann et al., 2011). Thermocycling conditions consisted of a 3 min initial denaturation at 98°C, 25 cycles of 98°C for 10 s, 53°C for 10 s, and at 72°C for 15 s, and a final extension at 72°C for 7 minutes (Eiler et al., 2012). For plastid-specific 23S rRNA gene sequencing, DNA was amplified using barcoded primer A with template specific primer p23SrV_f1 and primer B with template specific primer p23SrV_r1 (Sherwood and Presting, 2007; Steven et al., 2012). Thermocycling conditions consisted of a 2 min initial denaturation at

98°C, 25 cycles of 98°C for 10 s, 58°C for 10 s, and at 72°C for 15 s, and a final extension at 72°C for 10 minutes (Sherwood and Presting, 2007). PCR products from 2-3 reactions were combined and Qiagen Buffer EB was added to a final volume of 100 µL. To remove excess nucleotides and salts, 65 µL of Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA) were added and the manufacturer's protocol was followed. DNA was eluted from AMPure beads using 15 µl of Qiagen Buffer EB. Concentration of DNA for each sample was determined using the Invitrogen Qubit ds DNA HS assay and Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Amplicons were mixed in equimolar concentrations and processed from emPCR through sequencing following the May 2011 Roche emPCR Method Manual on a Roche GS FLX+ system with 2.8, flow pattern A, and analyzed through amplicon signal processing using Roche software version 2.8 at the Keck Center for Functional Genomics at the University of Illinois.

Using MacQIIME (Caporaso et al., 2010), 454-pyrosequencing sequence reads were assigned to samples based on barcode sequences and sorted into operational taxonomic units (OTUs) using the uclust method at a 0.97 similarity threshold (Edgar, 2010). A representative set of 16S rRNA gene sequences for each OTU were classified independently using the freshwater database (12 July 2012) compiled by the McMahon and Bertilsson research groups (Newton et al., 2011) and the Greengenes database (9 September 2011)(DeSantis et al., 2006) as taxonomy files for the classify.seqs command in mother (version 1.25.0) (Schloss et al., 2009). Plastid-specific 23S rRNA sequences were identified using the SILVA large subunit reference database (Quast et al., 2013) as a training set for the assign_taxonomy.py command in QIIME (Wang et al., 2007).

Statistical approach

Pairwise Bray-Curtis similarities were calculated for every combination of samples using Hellinger-transformed data for each community composition dataset (ARISA, 16S tag-pyrosequencing, 23S plastid tag-pyrosequencing). Non-metric multidimensional scaling (MDS) was used to display ARISA and 23S plastid tag-pyrosequencing Bray-Curtis similarities in multidimensional space using PRIMER version 6 (PRIMER-E Ltd, Plymouth Marine Laboratory, UK) (Clarke and Warwick, 2001). Permutational multivariate analysis of variance (PERMANOVA) was used to test: 1. the effects of light and temperature on bacterial community composition (ARISA) following incubation for each combination of bacteria and phytoplankton, 2. the effect of each phytoplankton treatment (compared to the no phytoplankton control) on bacterial community composition (ARISA) at each light and temperature level, stratified by the bacterial community source lake, 3. the effects of light and temperature on phytoplankton community composition (23s rRNA plastid tag-pyrosequencing) for each combination of bacteria with a phytoplankton assemblage. PERMANOVA is a non-parametric multivariate analysis of variance that generates p-values using permutations (Anderson, 2001; McArdle and Anderson, 2001). PERMANOVA tests were run using the adonis function from the vegan package (Oksanen et al., 2011) in the R statistical environment (R core development team, 2010).

The linear model function in the vegan package was run in the R statistical environment to identify 16S rRNA tag-pyrosequencing OTUs that responded to treatments. To test for effects of phytoplankton treatments on the abundance of each OTU, the linear model [OTU abundance] ~ [time]*[phytoplankton treatment] was tested for each phytoplankton treatment and corresponding control (e.g., TB bacteria + TB phytoplankton and TB bacteria + no

phytoplankton) for each combination of light and temperature. To test for effects of light and temperature on the abundance of each OTU, the linear model [OTU abundance] ~ [temperature]*[light] was run for each combination of bacteria and phytoplankton (e.g., TB bacteria + TB phytoplankton, SSB bacteria + no phytoplankton). For each combination of samples, the rrarefy function was used to resample DNA sequences generated from each sample so that each had the same number of sequences.

RESULTS

Effects of phytoplankton, temperature, and light on bacterial community composition

Over the five-day incubation, phytoplankton presence and composition, temperature, and light affected bacterial community composition (Fig. 4.2). Effects of light and temperature on bacterial community composition depended on both the phytoplankton treatment and bacterial community source (Fig. 4.3A). Direct effects of light and temperature on bacterial communities were inferred through the changes in bacterial community composition in no-phytoplankton control treatments. Temperature had a significant direct effect on community composition of bacteria from SSB, but only a marginally significant effect on TB bacteria (Fig. 4.3A). In contrast, light had a significant direct effect on the composition of bacterial communities from both lakes (Fig. 4.3A). When bacteria were incubated with phytoplankton, community change from initial composition generally increased with increased temperature (Fig. 4.2). Temperature had a significant effect on the composition of bacterial communities incubated with phytoplankton (Fig. 4.3A). Notably, the variation explained by temperature was consistently higher for bacteria incubated with phytoplankton from the "away" lake than for bacteria incubated with phytoplankton from their "home" lake (Fig, 4.3A).

The effect of phytoplankton treatment was evaluated relative to the corresponding no-phytoplankton controls, and the strength of the phytoplankton treatment effect depended primarily on temperature, but also on light. Prior to incubation, 22% of the variation in bacterial community composition in SSB phytoplankton and corresponding control treatments was explained by phytoplankton (Fig. 4.3B). Following incubation, variation explained by SSB phytoplankton was slightly higher in low light treatments, and only greater than the initial explained variation in microcosms incubated at the highest temperature (45% variation explained. In contrast, variation in bacterial community composition in TB phytoplankton and corresponding control treatments due to phytoplankton treatment was not significant prior to incubation ($p>0.05$; Fig. 4.3C). At the coldest two temperatures, 13% and 19% more variation in bacteria community composition was explained by TB phytoplankton in low light compared to high light treatments. As temperature increased, the variation explained by TB phytoplankton generally increased up to temperature 4 and differences in variation explained due to light treatment decreased.

Effect of phytoplankton composition on bacterial community composition

Microcosm phytoplankton assemblages were characterized via 454 tag-pyrosequencing of the 23S rRNA gene using plastid specific primers to determine whether effects of light and temperature on bacteria in phytoplankton treatments were due to shifts in phytoplankton composition. Light and temperature explained less variation in phytoplankton composition than they did for bacterial community composition (Fig. 4.4). Using microcosm sample scores on NMDS axes 1 and 2 (Fig. C.2) as a proxy for phytoplankton composition, the BIO-ENV procedure was used to identify the combination of NMDS axis 1, NMDS axis 2, temperature,

and light that was most correlated with changes observed in bacterial community composition. For each combination of bacteria and phytoplankton, temperature was most correlated with changes in bacterial community composition (Table 4.1). The second most correlated set of variables was temperature + light for both bacterial communities incubated with TB phytoplankton and temperature + one of the phytoplankton NMDS axes for bacterial communities incubated with SSB phytoplankton treatment.

Bacterial OTU response to phytoplankton, temperature, light

Tag-pyrosequencing of 16S rRNA genes in initial samples and post-incubation samples from the warmest (~25°C) and coldest (~10°C) temperatures was used to identify bacterial OTUs that differed in relative abundance due to experimental treatments. Phytoplankton treatment, temperature, and light conditions affected the relative abundance of OTU 2411, identified to the betaproteobacterial *Polynucleobacter* PnecC tribe and the most abundant OTU in the dataset, and OTU 1347, identified as bacVI-A (Fig. 4.5A, 4.5B). OTU 400, identified to the betI-A clade, increased in relative abundance in phytoplankton treatments while decreasing in corresponding no phytoplankton controls at the warmest temperature (Fig. 4.5C). Also contributing to observed phytoplankton effects, OTU 1805, identified to the *Chlamydiales* order, was only detected in post-incubation TB phytoplankton microcosms incubated at the warmest temperature and was more abundant in microcosms incubated at high light than low light levels (Fig. 4.5D).

Eighteen OTUs in addition to OTU 400 increased or decreased in phytoplankton treatments while exhibiting the opposite response in the corresponding no phytoplankton controls (Fig. 4.6). Bacterial OTUs that were positively enriched by phytoplankton included representatives of *Actinobacteria*, *Bacteriodetes*, *Alphaproteobacteria*, *Betaprotobacteria*,

Gammaproteobacteria, and *Verrucomicrobia*. In particular, three OTUs identified to the betaproteobacterial *Limnohabitans* genus (Lhab-A1, Lhab-A3, Lhab-A4) exhibited positive responses to phytoplankton. Bacterial OTUs that were depleted in relative abundance in phytoplankton treatments relative to no phytoplankton controls included *Actinobacteria*, *Betaproteobacteria*, and *Verrucomicrobia*. In addition to OTUs with significant responses to phytoplankton treatments, six OTUs, including OTU 1347, exhibited significant responses to temperature or light in at least two bacteria and phytoplankton treatment combinations.

DISCUSSION

Effects of phytoplankton, temperature, and light on bacterial community composition

Phytoplankton and environmental conditions affect bacterial community composition both directly and in combination. Direct effects of light and temperature on bacterial communities were inferred through the changes in bacterial community composition in no-phytoplankton control treatments. Light had a consistent direct effect on bacterial community composition. The effect of light may be due to selection for phototrophic bacteria (Zubkov, 2009; Martinez-Garcia et al., 2012). Alternatively, UV radiation may contribute to observed light effects. The microcosm bottles were made of borosilicate glass, which is opaque to UVB and shorter wavelengths but partially transparent to UVA (Döhring et al., 1996). UV light can have a direct negative effect on bacteria or affect bacteria indirectly through photodegradation of dissolved organic matter, which results in high concentrations of low molecular weight dissolved organic carbon and oxidative stress (Hortnagl et al., 2011; Paul et al., 2012; Santos et al., 2012).

Direct effects of temperature were observed for SSB bacteria, but not TB bacteria. In no-phytoplankton control microcosms containing TB bacteria, composition of bacterial communities

incubated in the high light treatment changed more due to temperature than those in the low light treatment. These observations suggest that lack of a significant temperature response for TB bacteria incubated without phytoplankton may be due to limited availability of labile dissolved organic matter. Similar to the direct effect of temperature observed for SSB bacteria, temperature affected the composition of bacterial communities collected from the inlet and outlet of an arctic lake following a five-day incubation without phytoplankton (Adams et al., 2010). The artic bacteria temperature manipulation experiment used a similar temperature range (8-20 °C), but differed from the current experiment in that it was a regrowth experiment where bacterial abundance started at 50% of ambient levels (compared to ~95% of ambient levels used in the current experiment), and the arctic incubations were carried out in the dark.

Effects of temperature were primarily mediated by phytoplankton, and the effects of phytoplankton on bacterial community composition depended on light and temperature conditions. Changes in phytoplankton composition over the course of the experiment do not appear to explain phytoplankton-mediated effects of temperature, as temperature alone was more correlated with observed changes in bacterial communities than any combination of variables that included NMDS axes representing differences in phytoplankton community composition (Table 4.1). As temperature increased in microcosms that contained phytoplankton assemblages, change in bacterial communities from their initial composition increased. The amplification of community change with increased temperature is likely due to increased enzyme activity and process rates of both phytoplankton and bacteria (Overmann, 2013). Temperature consistently explained more variation in bacterial community composition when bacteria were incubated with "away" phytoplankton that originated in the opposite lake than when incubated with "home" phytoplankton. Since phytoplankton community composition affects bacterial community

composition in lakes as demonstrated in Chapter 2 (Paver et al., 2013), bacterial communities have already been shaped by phytoplankton from their "home" lake. Previous exposure to "home" phytoplankton makes incubations with "home" phytoplankton less able to induce changes in bacterial community composition than "away" phytoplankton, which potentially exposes bacterial communities to novel phytoplankton-derived organic matter (Hellebust, 1965; Fogg, 1983; Sarmento and Gasol, 2012).

Additional insight into how phytoplankton and bacteria interact can be inferred from changes observed under different light and temperature conditions. Variation in bacterial community composition explained by TB phytoplankton depended on light and temperature conditions. At the warmest temperature, the variation explained by phytoplankton was greater for high light than low light treatments. In contrast, at the coldest temperature, the variation explained by phytoplankton was greater for low light than high light treatments. We hypothesize that the enhanced effect of phytoplankton on bacterial community composition at high light levels at the warmest temperature is due to light-dependent differences in the amount and composition of exudates produced. Concentration of extracellular organic carbon excreted from cultures of phytoplankton has been shown to increase with increasing light levels and to increase with temperature up to a species-specific temperature optimum (Zlotnik and Dubinsky, 1989). Glycolate, a common algal exudate and product of photorespiration, is released at high light levels, especially when temperatures are warm (Parker and Armbrust, 2005). The increased effect of phytoplankton on bacterial community composition at low light levels compared to high levels at the coldest temperature may be due to increased grazing pressure. Increased rates of bacterial ingestion with reduced light levels has been demonstrated for the chrysophytes *Dinobryon cylindricum* (Caron et al., 1993) and *Poterioochromonas malhamensis* (Holen, 1999).

Differences between light levels were greatest at the coldest temperature for "home" phytoplankton treatments, and greatest at the warmest temperature for "away" phytoplankton treatments. This observation is consistent with the enhanced effect of phytoplankton-derived dissolved organic matter, including exudates, on "away" bacterial assemblages for whom these resources are novel, and the increase in light-dependent effects at warmer temperatures (Zlotnik and Dubinsky, 1989; Parker and Armbrust, 2005). In the absence of novel resources, compositional changes in "home" bacterial communities may be particularly sensitive to grazing effects. This hypothesis is consistent with the observation that the effect of the light treatments for "home" bacterial communities was most pronounced at the coldest temperature, and that grazing was previously hypothesized to be especially influential at low temperatures.

Bacterial OTU response to phytoplankton, temperature, light

Responses of OTUs identified by 16S rRNA tag-pyrosequencing provide insight into the ecology of different groups of freshwater bacteria. *Polynucleobacter* is a cosmopolitan and abundant freshwater bacterial genus (Hahn, 2003; Jezberová et al., 2010; Newton et al., 2011). OTU 2411, identified to the PnecC subgroup of *Polynucleobacter*, was the most abundant OTU detected in the dataset and average relative abundance of replicates of each treatment following incubation ranged from 2.5 to 22.5%. OTU 2411 decreased in relative abundance in all treatments at the warmest temperature (Fig. 4.5A). When incubated without phytoplankton at the coldest temperature, OTU 2411 increased in relative abundance in high light microcosms, and also increased in relative abundance when the SSB bacteria were incubated at low light. In a previous study, *Polynucleobacter necessarius* ssp. *asymbioticus* (PnecC) lineage F10 was shown to increase in abundance faster when visible light was available than in the dark and even faster

when UV radiation and visible light was available (Hahn et al., 2012), supporting our observation of enhanced growth at the highest light level. Increased growth under high light levels may be due to an increase in low molecular weight substrates derived from photooxidation of humic substances, which have been proposed to sustain the growth of the PnecC F10 lineage (Hahn et al., 2012). OTU 2411 may also have gained a competitive edge under high light conditions through aerobic anoxygenic photoheterotrophy. In a study of genes for photoheterotrophy in single amplified genomes, *Polynucleobacter* were the dominant aerobic anoxygenic photoheterotrophs in freshwater lakes (Medina-Sanchez et al., 2006). Increased relative abundance of *Polynucleobacter* observed when bacteria were incubated in control treatments was not observed when bacteria were incubated with SSB phytoplankton. Bacteria within the *Polynucleobacter* genus have been shown to have lineage-specific responses to phytoplankton (Chapter 3). It has also been observed that when grown without algae, the percent of PnecC lineage F10 in the total bacterial community was fairly consistent throughout a fifteen-day batch culture experiment (Hahn et al., 2012). However, the percent of PnecC bacteria decreased precipitously in the first five days when incubated with *Dinobryon* sp,, a known mixotrophic phytoplankton genus (Hahn et al., 2012), suggesting that grazing by *Dinobryon* sp. or phytoplankton-induced increases in the abundance of other bacteria may affect the relative abundance of PnecC.

While not detected frequently in the freshwater 16S rRNA gene database (Newton et al., 2011), bacteria in the *Bacteriodetes* bacVI-A Muci tribe (*Mucilaginibacter* genus) increased over the incubation in many treatments in the current study as well as the algal exchange experiment described in Chapter 2 (Paver et al., 2013). Bacteria in the *Mucilaginibacter* genus have been described as non-motile rods producing large amounts of extracellular polymeric substances

(Pankratov et al., 2007) and isolates have been cultured from wetland freshwater (Baik et al., 2010), an acidic *Sphagnum* peat bog (Pankratov et al., 2007), and the rhizosphere of an herb (Kim et al., 2010). The effects of light and temperature on the abundance of Muci tribe OTUs 1347 and 3072 were significant and depended on the phytoplankton treatment. OTU 1347 abundance was generally higher following incubation at high light compared to low light, suggesting that these bacteria may be phototrophic. Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) genes have been found in single amplified genomes of other Sphingobacteria (Martinez-Garcia et al., 2012). When incubated with SSB phytoplankton at the coldest temperature, OTU 1347 abundances were similar to those observed in no-phytoplankton microcosms and higher than the abundances observed at high temperatures. OTU 1347 may have been out competed for resources at the highest temperature or experienced losses due to grazing pressure from mixotrophic phytoplankton (Wilken et al., 2013).

Findings from this study provide additional evidences for a positive relationship between bacteria in the cosmopolitan freshwater clade betI-A (*Limnohabitans* genus) and phytoplankton (Newton et al., 2011). OTUs representing betI-A tribes Lhab-A1, Lhab-A3, and Lhab-A4 were all enriched by phytoplankton. OTU 400 was identified to the betI-A clade, but its tribe affiliation could not be determined. Organisms in the betI-A clade were enriched the algal exchange microcosm experiment described in Chapter 2 (Paver et al., 2013), found to be abundant in algal cultures (Šimek et al., 2011), and observed to co-occur with periods of high extracellular phytoplankton production (Šimek et al., 2008). Bacteria in the betI-A clade are also known for their ability to respond quickly to changes in environmental conditions due to relatively short population turnover times (Šimek et al., 2001; Šimek et al., 2005; Šimek et al., 2006; Alonso et al., 2009). They are additionally highly susceptible to grazing, and increase their

growth rates in the presence of flagellates (Šimek et al., 2001; Hornák et al., 2005; Šimek et al., 2005; Weinbauer et al., 2007).

Bacteria in the phylum Chlamydiae went from being rarely detected in pre-incubation samples to making up 2-13% of bacterial sequences in microcosms with TB phytoplankton incubated at the warmest temperature. The increase in Chlamydiae was due to OTU 1805, which was identified to the order *Chlamydiales*. Chlamydiae have an obligately intracellular lifestyle with two distinct stages: a metabolically inactive elementary body that exists outside of a host cell and can infect a host cell and a metabolically active reticulate body that multiples inside the host (Corsaro et al., 2003). Release of free-living stage Chlamydiae would contribute to the 20-80% of metabolically inactive bacteria observed in the environment (Cole, 1999). Presence of inactive Chlamydiae in the water column would also contribute to observations of dormancy as defined by bacteria that are underrepresented in RNA-based analyses compared to DNA-based analysis (Jones and Lennon, 2010). The relationship between Chlamydiae and their documented hosts, which range from amoebae to humans, include endosymbionts, parasites, and agents of infection (Corsaro et al., 2003). Diverse environmental *Chlamydiales* have been detected at abundances up to 5×10^4 cells per millileter from the water column of a shallow mesoeutrophic lake (Pizzetti et al., 2012) and recovered from cocultures with freshwater amoeba (Corsaro and Venditti, 2009).

Limitations

There are a few limitations with the study design that merit discussion. Due to the way microcosms were assembled, the effects of initial bacterial community composition and differences in lake chemistry are confounded. Consequently, it is not possible to determine

whether differences in the response of bacterial communities from different lakes to various treatments is due to the organisms present in each community or differences in lake chemistry. A second limitation is that size fractioning of native phytoplankton assemblages has the potential to include rotifers in the 20-100 μm size range (Paver et al., 2013). As a result, it is not possible to decisively conclude that the host for *Chlamydiales* OTU 1805 is a phytoplankton. Finally, the relative-abundance based molecular methods used to characterize bacterial community composition are not quantitative and may lead to misinterpretation of OTU responses. For example, an OTU may appear to decrease in relative abundance while maintaining a consistent population size or even increasing in abundance if other populations are increasing more rapidly.

Conclusion

Light and temperature influence bacterial communities directly as well as by altering interactions between phytoplankton and bacteria. Light availability directly affected bacterial community composition, potentially due to stimulating the growth of phototrophic bacteria. Effects of phytoplankton and temperature were highly interdependent. When bacteria were incubated with phytoplankton, similarity of bacterial communities to their initial composition decreased as temperature increased. The effect of temperature was greatest for "away" phytoplankton treatments. We hypothesize that the enhanced effect of "away" phytoplankton on bacteria is due to the availability of a different pool phytoplankton-derived resources than are available in their "home" lake. At low temperatures, predation on bacteria by mixotrophic phytoplankton may additionally contribute to the effect of phytoplankton on bacterial community composition. Change in relative abundance of different bacterial OTUs due to incubation with different phytoplankton treatments under a range of environmental conditions additionally

provided insight into bacterial traits and adaptations. Further investigation into the mechanisms of phytoplankton influence and a trait-based understanding of bacterial taxa will be instrumental in developing a predictive framework for bacterial community temporal patterns. Such a framework will be useful for examining mechanisms that create and maintain diversity and enhance our ability to forecast community responses to environmental change.

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TABLES

Table 4.1. Correlations between the 16S rRNA pyrosequencing Bray-Curtis similarity matrix and Euclidean distance matrices of environmental variables (temperature, light) and phytoplankton treatment variables (SSB vs .TB, NMDS axis 1 scores, NMDS axis 2 scores) determined by the BEST algorithm.

	Phytoplankton	SSB		TB	
Explanatory variables	Bacteria	SSB	TB	SSB	TB
Temperature		0.853	0.847	0.861	0.821
Temperature + light		0.577	0.630	0.602	0.677
Temperature + NMDS axis 2		0.342	0.558	0.762	0.543
Temperature + NMDS axis 1		0.756	0.503	0.571	0.390

FIGURES

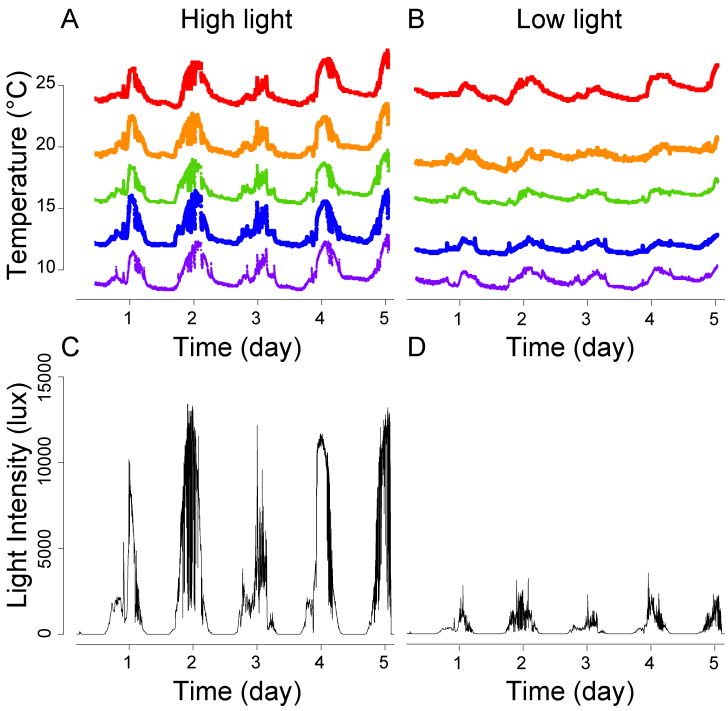

Figure 4.1. Temperature (A, B) and average light intensity (C, D) at the top (High light; A, C) and bottom (Low light; B, D) of each temperature incubator over the five-day incubation.

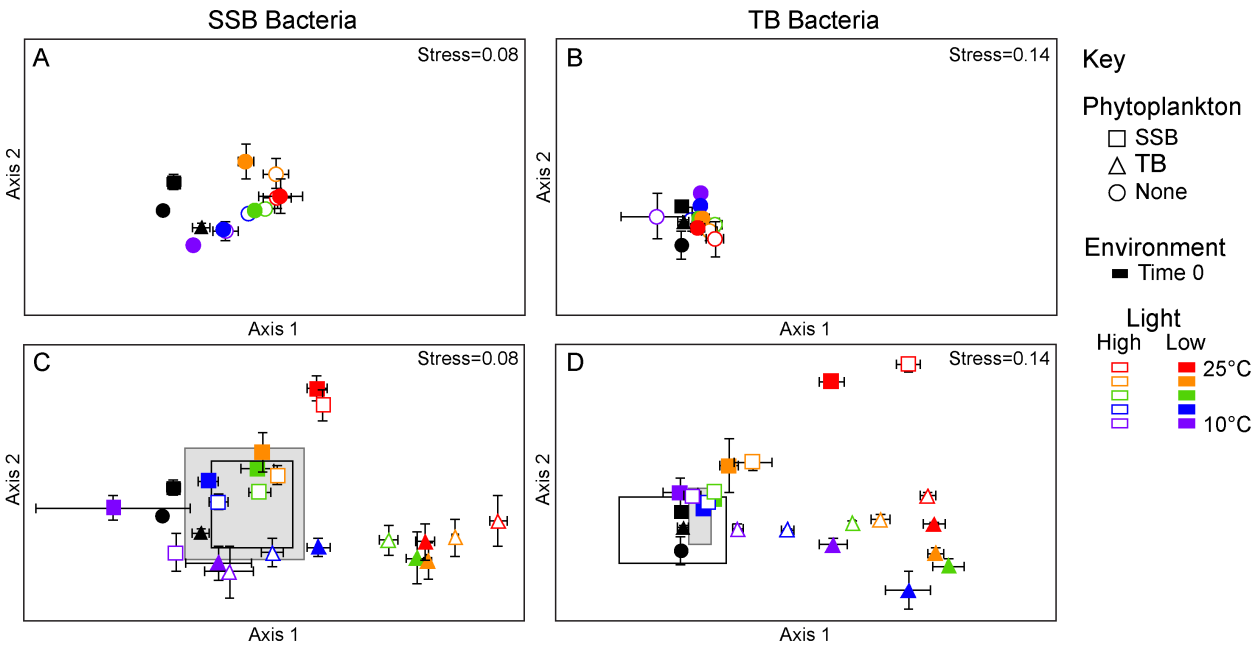

Figure 4.2. Non-metric multidimensional scaling ordination of bacterial community composition in microcosms (average ± standard error) with SSB bacteria (A and C) and TB bacteria (B and D) before and after the five day incubation. To simplify depiction of overlapping treatments, community composition in no-phytoplankton control microcosms following incubation is shown in plots A and B and the range of bacterial community composition observed for high light and low light no phytoplankton controls is indicated by a black outline and a grey rectangle, respectively in plots C and D. Community composition before incubation (Time 0) is included in all plots. When bacteria were incubated with phytoplankton, similarity to initial community composition decreased with increased temperature and the trajectory followed depended on the phytoplankton source lake. Light effects were evident, but not as pronounced as the effects of phytoplankton and temperature.

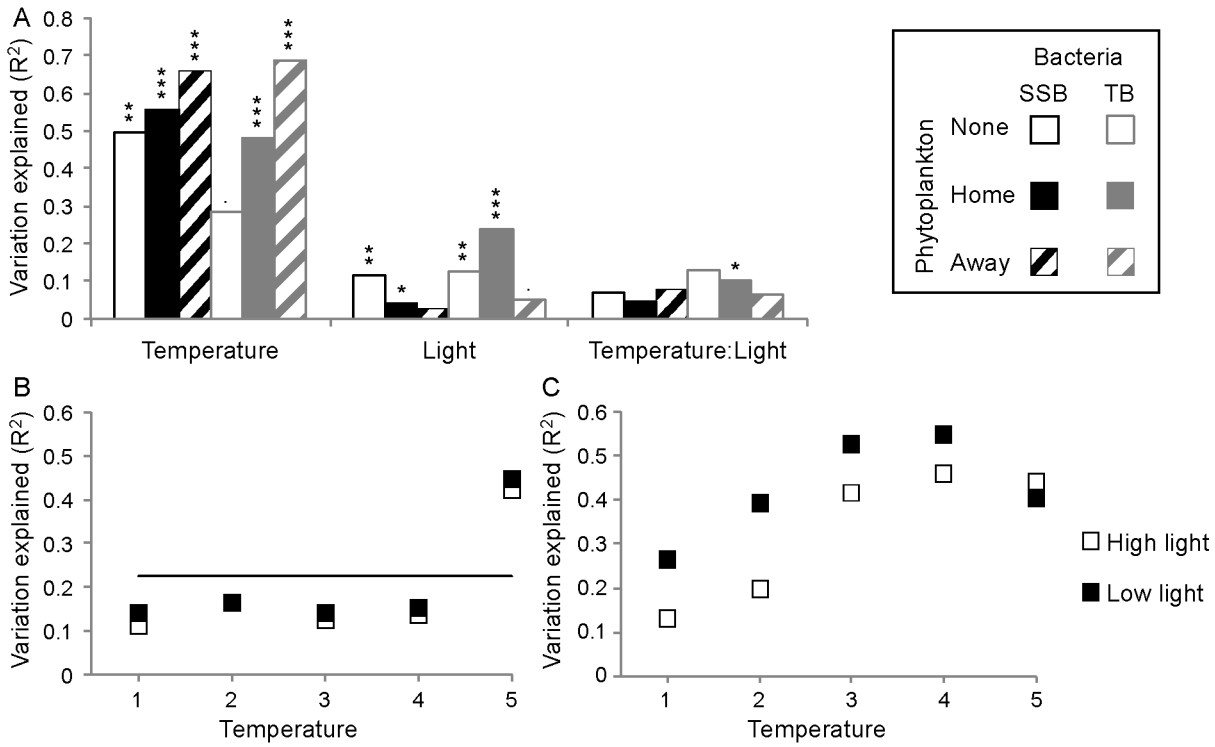

Figure 4.3. Variation in bacterial community composition explained by (A) temperature, light, and the interaction between light and temperature for each combination of phytoplankton and bacteria, (B) SSB phytoplankton and (C) TB phytoplankton at different light and temperature levels as determined by PERMANOVA. The analysis of the effect of phytoplankton (B and C) was stratified to remove the effect of bacterial source. The SSB phytoplankton treatment explained a significant amount of variation prior to incubation and a line indicating the pre-incubation variation explained is included in plot B for reference. A: *** $p<0.001$, ** $p<0.01$, * $p<0.05$, . $p<0.1$; B and C: high light temperature 1 $p<0.05$, all other treatments $p<0.01$.

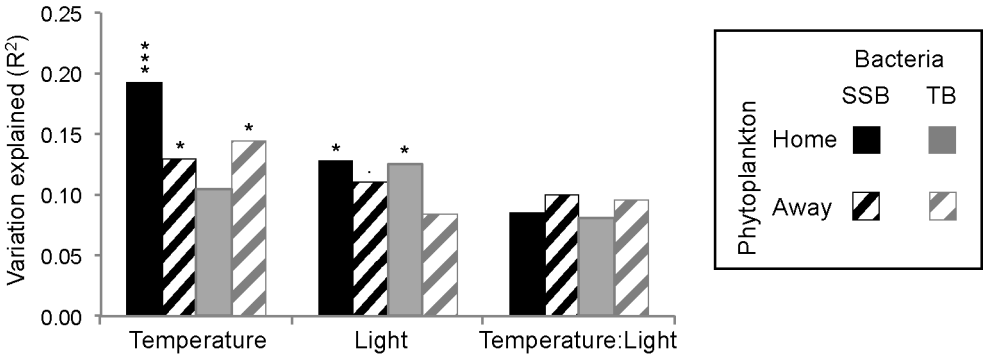

Figure 4.4. Variation in phytoplankton community composition determined by 23S rRNA plastid pyrosequencing explained by temperature, light, and the interaction between light and temperature for each combination of phytoplankton and bacteria. Symbols: *** $p<0.001$, ** $p<0.01$, * $p<0.05$, . $p<0.1$.

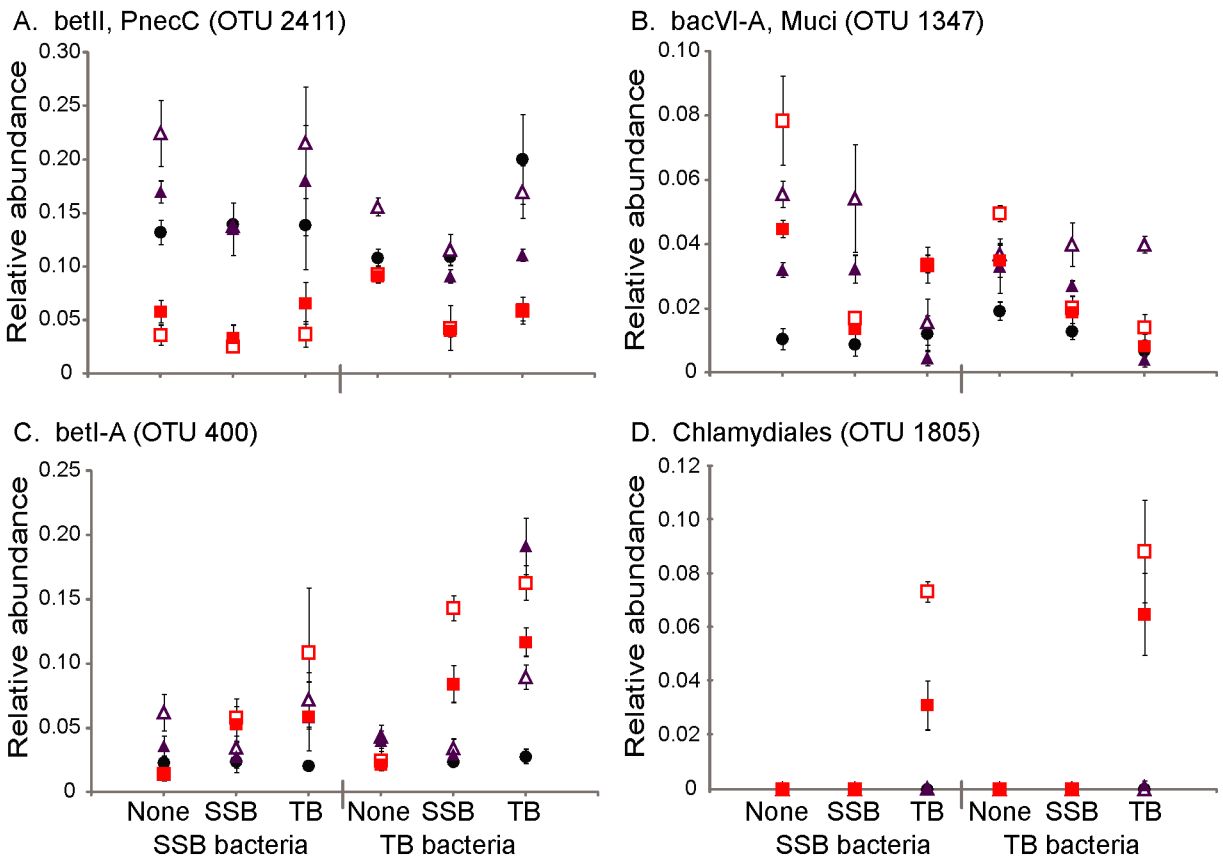

Figure 4.5. Relative abundance of abundant bacterial OTUs that contribute to differences observed among environmental treatments (A and B) and between phytoplankton and no phytoplankton controls (C and D) before incubation (- ●-) and following incubation in cold and low light (- ▲-), cold and high light (- △-), warm and low light (- ■-), and warm and high light (-□-) environments (average ± standard error).

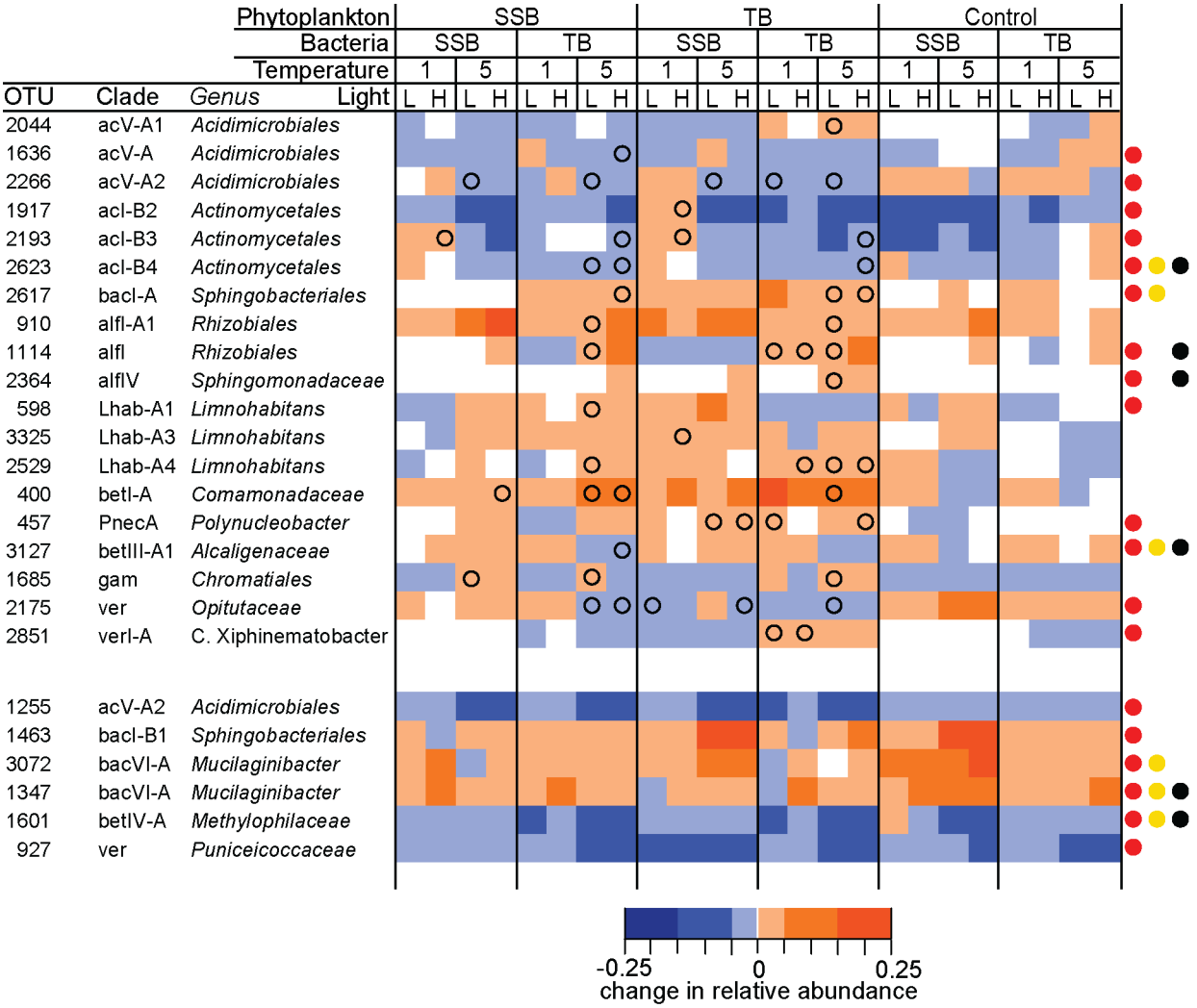

Figure 4.6. Change in relative abundance over the five-day incubation (final relative abundance –initial relative abundance) of (A) bacterial OTUs significantly enriched or depleted in at least one phytoplankton treatment (SSB, TB) relative to the corresponding no-phytoplankton control and (B) bacterial OTUs that responded significantly to temperature, light, or the interaction between light and temperature in multiple bacteria and phytoplankton treatments. Open circles indicate the phytoplankton treatments that differed from the corresponding no-phytoplankton control for each OTU. Colored circles to the right of the heatmap indicate whether OTU abundance was affected by temperature (red), light (yellow), or the interaction between light and temperature (black). OTUs were identified using the naming scheme described by Newton et al. (2011) and Linnaean classification to the finest level of classification with at least 90% assignment confidence. Abbreviations: C., Candidatus; alf, *Alphaproteobacteria*; bet, *Betaproteobacteria*; gam, *Gammaproteobacteria*; ac, *Actinobacteria*; bac, *Bacteroidetes*; ver, *Verrucomicrobia*.

CHAPTER 5

TEMPORAL SUCCESSION OF PUTATIVE GLYCOLATE-UTILIZING BACTERIOPLANKTON TRACKS CHANGES IN DISSOLVED ORGANIC MATTER IN A HIGH-ELEVATION LAKE[1]

ABSTRACT

Shifts in the composition of dissolved organic matter (DOM) may be a principal factor effecting changes in bacterial community composition. Emerald Lake, a high elevation lake with DOM dominated by terrestrial sources in the spring with increasing phytoplankton-derived inputs throughout the ice-free season, provided a natural experiment with which to investigate the importance of resource-mediated drivers. Glycolate-utilizing bacteria, a subset of the bacterial community able to use algal exudates, were characterized using DNA analysis of glycolate oxidase subunit D (*glcD*) genes from Emerald Lake samples collected approximately biweekly from ice-cover (June) through fall turnover (September). *glcD* genes were not detected in early season samples when the lake was ice-covered and phytoplankton-derived resources were scarce. Following this period, *glcD* gene composition exhibited significant changes through time, which were strongly correlated with the combination of fluorescence index, an indicator of the proportion of lake vs. terrestrially derived DOM, and dissolved inorganic nitrate+nitrite. These results suggest that seasonal shifts in dissolved organic matter source and environmental variables that affect the composition of algal-derived dissolved resources drive changes in the

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Author contributions: SFP, CEN, and ADK designed research. CEN collected samples and extracted DNA. SFP carried out *glcD* sequencing and T-RFLP. SFP analyzed data and wrote paper with input from CEN and ADK.

relative abundance and composition of putative glycolate-utilizing bacteria. These findings strengthen the evidence for shifts in dissolved organic matter structuring bacterial communities.

INTRODUCTION

Seasonal changes in the source and composition of dissolved organic matter may be a principal factor underlying succession in lake bacterioplankton communities (Crump*, et al.*, 2003, Kent*, et al.*, 2007, Nelson, 2009). Shifts in bacterial community composition have been observed following transitions from primarily terrigenous to more autochthonous (within lake) sources of organic matter (Crump*, et al.*, 2003, Nelson, 2009). Seasonal development of the phytoplankton community in lakes corresponds with changes in the quantity and composition of algal-derived substrates (Charpin*, et al.*, 1998) and has also been correlated with changes in bacterial community composition (Kent*, et al.*, 2007). Comparisons of bacterial community composition across lakes with different relative contributions of terrestrial-derived and phytoplankton-derived dissolved organic matter (Jones*, et al.*, 2009), as well as bacterial community shifts induced by addition of organic matter from different sources (Judd*, et al.*, 2006, Kritzberg*, et al.*, 2006, Perez & Sommaruga, 2006) further indicate the potential for organic matter resource partitioning to affect bacterial community composition. Changes in bacterial community composition associated with shifts in dissolved organic matter may also lead to changes in community function, such as denitrification potential (Ishida*, et al.*, 2008) or bacterial production (Judd*, et al.*, 2006). Characterizing the response of bacteria that are capable of utilizing particular resources to changes in dissolved organic matter may confirm the importance of resource-mediated drivers for shaping bacterial community composition and

enhance our ability to predict bacterial community response to changes in dissolved organic matter.

Bacteria able to use algal exudates are of particular relevance for studying resource-mediated drivers. Phytoplankton in lakes release on average 5-41% of primary productivity as dissolved organic substrates, and extracellular release of these products has a significant positive correlation with chlorophyll *a* concentration (Baines & Pace, 1991). Extracellular release products include algal exudates, which are substrates released by healthy algal cells such as carbohydrates (e.g. glucose) and amino acids (e.g. glycine) (Fogg, 1983). Bacteria rapidly utilize algal exudates (Cole*, et al.*, 1982). Exudate release during the day has been proposed to explain observed daily accumulation of dissolved organic carbon, a corresponding increase in community respiration, and subsequent overnight drawdown of accumulated carbon (Sadro*, et al.*, 2011b). Characterizing bacteria that utilize algal exudates can be challenging, however, because many algal-derived substrates in a lake (e.g. glucose) can have multiple origins from both allocthonous and autochthonous organic matter sources.

Glycolate-utilizing bacteria are a subset of the total bacterial community that can be specifically linked to algal exudates, and thus autochthonous resources. Glycolate is a product of photorespiration, commonly released as an exudate by various species of phytoplankton in both freshwater and marine systems (Hellebust, 1965, Fogg, 1983, Oliver, 1998). Because glycolate is rapidly utilized after extracellular release (Wright, 1975), it is unlikely that glycolate available in pelagic habitats would originate from outside of the lake. The diversity and community structure of bacteria with the genetic potential to use glycolate can be characterized using the *glcD* gene, which encodes the D subunit of the enzyme glycolate oxidase (Lau & Armbrust, 2006). Changes in the composition of *glcD* genes have been correlated with changes in the phytoplankton

community composition in lakes (Paver & Kent, 2010). Additionally, during the succession of a marine phytoplankton bloom, some *glcD* phylotypes were observed to increase in abundance, while the abundance of other phylotypes remained constant or decreased (Lau*, et al.*, 2007). In both freshwater and marine studies where putative glycolate-utilizing assemblages were observed through time, reported chlorophyll *a* levels indicate that autochthonous resources were consistently available (Baines & Pace, 1991, Kent*, et al.*, 2007, Lau*, et al.*, 2007).

This study sought to clarify potential mechanisms underlying resource-mediated drivers in lakes by determining how the composition of bacteria that use algal resources change as algal-derived resources transition from scarce to abundant. To achieve this objective, temporal patterns in the composition of putative glycolate-utilizing bacteria, a subset of bacteria able to use algal resources, were characterized in Emerald Lake, which exhibits a pronounced seasonal shift in organic matter from terrestrial sources in the spring to increasing phytoplankton-derived dissolved organic matter (DOM) throughout the ice-free season (Sadro*, et al.*, 2011a). The shift in DOM source has previously been correlated with annually recurring changes in total bacterial community composition (Nelson, 2009). It has additionally been demonstrated that bacteria in Emerald Lake rapidly utilize dissolved organic matter produced during the day, a substantial component of which is likely made up of algal exudates (Sadro*, et al.*, 2011b).

METHODS

Study site and sample collection

This study was conducted in Emerald Lake, a dimictic high-elevation (2800 m above sea-level) headwater cirque lake located on the western slope of the south central Sierra Nevada (California, CA, USA, 36°135’49” N, 118°40’30” W). Water samples were collected

approximately biweekly throughout the ice-free season from 3-4 depths in the center of the lake (0, 2, 4 and 8 m) and from the main inlet, which carries terrestrial runoff into the lake. Sample collection began on 6 June 2005 when the lake was ice-covered, and continued through snowmelt, ice-off, and fall turnover to 18 September 2005.

Sample collection and processing were described in detail by Nelson (2009). Briefly, each sample was filtered through 0.22 μm pore size polyethersulfone filter cartridge (Millipore Sterivex SVGP01050) to collect bacterial cells, which were preserved with sucrose lysis buffer and stored frozen prior to DNA extraction using silica centrifuge columns. A continuous record of thermal structure, meteorology, and outflow were used to derive thermal stability, buoyancy frequency maxima, and residence time (Nelson, 2009). Discrete environmental data collected at sampling intervals included the following: *in situ* temperature and dissolved oxygen at sampling depth, pH, chlorophyll *a* concentration, dissolved fluorescence index, specific UVA absorbance; particulate carbon, dissolved organic carbon; particulate nitrogen, total dissolved nitrogen, dissolved inorganic nitrate+nitrite; particulate phosphorus, total dissolved phosphorus, soluble reactive phosphorus. Fluorescence index indicates the relative contribution of terrestrial- and lake-derived organic matter to the lake's dissolved organic matter pool (McKnight et al., 2001). Details on the measurement of environmental parameters can be found in Nelson (2009).

glcD gene sequencing

Genes encoding the D subunit of glycolate oxidase (*glcD*) were amplified from DNA extracted from samples collected at 2 m on 7 July, 31 July, and 18 September 2005 using primers glcD-1f, 5'-GACCCAGACAATCGGAGTGCCGTGGTTSARCCNGGNGT-3' and glcD-2r, 5'-TGCATGTTTCCATCTCCTGCGTGRAANACRTT-3' (Lau and Armbrust, 2006). Polymerase

chain reaction conditions were described by Paver and Kent (2010). PCR products from two 50µl reactions were concentrated and purified using QIAquick PCR purification followed by gel extraction according to the manufacturers instructions (Qiagen, Valencia, CA, USA). Three clone libraries, one from each date, were then constructed using a pGEM-T Easy kit according to manufacturer instructions (Promega, Madison, WI, USA). Following MinElute PCR purification (Qiagen, Valencia, CA, USA), PCR products were sequenced by the W. M. Keck Center for Functional Genomics at the University of Illinois using ABI Prism BigDye terminator sequencing kit (Applied Biosystems Inc., Foster City, CA, USA) with standard PCR sequencing conditions using the M13 forward primer.

Sequence and phylogenetic analysis

Sequences were trimmed to contain the region between glcD-1f and glcD-2r primers using Geneious Pro 5.1.7 (Drummond et al., 2009b). One sequence not containing the conserved domain for *glcD*, COG0277, based on the NCBI database of Clusters of Orthologous Groups of proteins (Marchler-Bauer et al., 2009) was removed from subsequent analyses. DNA sequences from all clone libraries, 187 total, were aligned using Translation Align with default parameters of the MUSCLE protein alignment option in Geneious Pro 5.1.7 (Drummond et al., 2009a). An uncorrected-P distance matrix calculated from the DNA sequence alignment using SplitsTree version 4.8 (Hudson and Bryant, 2006) was analyzed by DOTUR (Schloss and Handelsman, 2005) to group DNA sequences into operational taxonomic units (OTUs) at the 99% DNA sequence similarity level. These sequence data have been submitted to GenBank under accession No. JQ517539-JQ517722.

A *glcD* amino acid tree was constructed using amino acid sequences translated from a representative DNA sequence for each OTU and the top two BLASTX hits for each OTU. Amino acid sequences were aligned using the MUSCLE align option with default parameters in Geneious Pro 5.1.7 (Drummond et al., 2009b). Columns with gaps present in the majority of sequences were removed. Maximum likelihood trees were constructed in PROML (Phylip version 3.65) using the Jones-Taylor-Thornton probability model, constant rate of change, and randomized sequence addition with 100 bootstrap replicates (Felsenstein, 2005). The majority rule algorithm was used to construct a consensus tree using Genious Pro 5.1.7. The UniFrac Significance analysis in Fast UniFrac was used to determine whether organisms were significantly clustered by sample (Hamady et al., 2010). All samples were analyzed together and 500 permutations were used to generate a p-value. A heatmap was created using the gplots package in R (R Development Core Team, 2010) to visualize OTU relative abundances within clone libraries.

glcD gene terminal restriction fragment length polymorphism (T-RFLP)

PCR amplification of *glcD* genes from samples collected at all depths on each of the eleven sample dates was carried out as described above with the glcD-2r primer labeled at the 5' end with the phosphoramidite dye 6-FAM. The concentration of template DNA was doubled for samples that did not produce visible bands when screened using gel electrophoresis. MinElute PCR purification (Qiagen, Valencia, CA, USA) was used to concentrate 3-4 50µl reactions and remove excess primers, nucleotides, and salts (Qiagen, Valencia, CA, USA). REPK (Collins and Rocap, 2007) was used to predict terminal fragment lengths for each Emerald Lake *glcD* sequence when cut by a number of different restriction enzymes, and *AluI* and *MboI* were

identified as the best pair of enzymes for differentiating OTUs present in this lake. Purified PCR products were digested in single-enzyme incubations containing *AluI* and *MboI* (New England BioLabs Inc., Ipswich, MA, USA). Digested PCR products were combined with the ABI GeneScan ROX 1000 size standard and analyzed by denaturing capillary electrophoresis using an ABI 3730XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the W. M. Keck Center for Functional Genomics at the University of Illinois. Size-calling, profile alignment, and automatic grouping of terminal restriction fragments into OTUs were carried out using GeneMarker version 1.75 software (SoftGenetics, State College, PA, USA). A peak threshold of 50 fluorescence units was used to include the maximum number of peaks while excluding background fluorescence. The area under each peak (signal strength) was normalized to the total area of all peaks in each electropherogram to account for run-to-run variations in signal detection, representing each peak as a proportion of the total fluorescence. Normalized data from each enzyme was concatenated for each sample (Fierer et al., 2003; Paver and Kent, 2010; Peralta et al., 2010).

Data analysis

Bray-Curtis similarity of *glcD* T-RFLP profiles was calculated for all sample pairs (Legendre and Legendre, 1998). Non-metric multidimensional scaling was used to visualize Bray-Curtis similarities among samples in a two-dimensional ordination. The pattern of similarities observed among *glcD* T-RFLP profiles was compared to the pattern of similarities observed among 16S rRNA gene T-RFLP profiles generated from the same samples (Nelson, 2009) using a non-parametric Mantel test (Clarke and Warwick, 2001). Differences in putative glycolate-utilizing assemblages through time and among depths was tested using a two factor

permutational multivariate analysis of variance (PERMANOVA). PERMANOVA is a non-parametric multivariate analysis of variance with p-values generated using permutations (Anderson, 2001; McArdle and Anderson, 2001). The adonis function from the vegan package (Oksanen et al., 2011) in the R statistical environment (R Development Core Team, 2010) was used to run PERMANOVA. Non-parametric partial Mantel tests comparing patterns of similarity in *glcD* T-RFLP and environmental parameters were conducted to evaluate relationships between community dynamics and potential ecological drivers in Emerald Lake (inlet samples were excluded from this analysis). The statistical procedures described above were carried out using the RELATE, ANOSIM, and BEST (BIO-ENV, BV-STEP) procedures in PRIMER 6 (PRIMER-E Ltd, Plymouth, UK). Local similarity analysis (LSA) was used to detect correlations between relative abundance of specific OTUs determined by T-RFLP and environmental factors from samples collected from the 2 m depth in Emerald Lake using the LSA code developed by Ruan and colleagues (2006) in the R statistical environment (R Development Core Team, 2010) as described by Paver and Kent (2010). Correlations were considered to be significant if the p-values associated with either the local similarity score or the Pearson correlation was less than 0.001. LSA networks were visualized in Cytoscape v. 2.6.1 (Shannon et al., 2003). Restriction fragments were identified as specific sequence-based OTUs by cross-referencing observed restriction fragment sizes with OTU fragment sizes predicted from *in silico* digests of *glcD* sequences.

RESULTS

Taxonomic diversity of glcD genes

At the 99% DNA sequence similarity level, *glcD* sequences formed twenty-five operational taxonomic units (OTUs) among the three dates from which genes were sequenced. The phylogenetic structure of the *glcD* assemblage changed significantly over time (UniFrac, p=0.002 for comparisons among the three clone libraries). In the *glcD* amino acid phylogeny, OTUs derived from Emerald Lake clustered with *glcD* sequences from *Alphaproteobacteria, Betaproteobacteria*, *Planctomycetes, and Acidobacteria* (Fig. 5.1). The majority of sequences (88%) were found within the *Betaproteobacteria*, with OTUs associating into two major clades. Nine OTUs representing 39% of total sequences formed a cluster containing the *glcD* sequence from *Polynucleobacter necessarius* with a sister group containing *glcD* sequences from *Ralstonia* sp. and *Burkholderia* sp. Eight OTUs, representing 47% of total sequences, formed a sister group to *Achromobacter* sp. and *Bordetella* sp. Within the *Planctomycetes*, a cluster of four OTUs representing 10% of total sequences formed a sister group to the *glcD* sequence from *Gemmata obscuriglobus.*

Temporal patterns of glcD genes

T-RFLP of *glcD* genes was carried out on samples collected biweekly from June through October. Samples collected from Emerald Lake from 6 June through 20 June and samples collected from the inlet prior to 25 August did not produce visible bands when analyzed with gel electrophoresis or detectable terminal restriction fragments when digested PCR products were analyzed with capillary electrophoresis, and could not be analyzed for diversity or composition of *glc*D gene sequences. The putative glycolate-utilizing bacterial assemblage as characterized

by *glcD* T-RFLP was temporally dynamic and was significantly correlated with patterns of change observed in the total bacterial community characterized by 16S rRNA gene T-RFLP (Nelson 2009) (RELATE ρ=0.65, p<0.001) (Fig. 5.2). Notably, snowmelt *glcD* assemblages exhibited high Bray-Curtis similarity with early ice-off assemblages (74% similarity; PERMANOVA R^2=0.240, p=0.557) while snowmelt total bacterial communities were distinct from ice-off communities (45% similarity; PERMANOVA, R^2=0.802, p=0.048). Conversely, community change during the transition between ice-off and fall turnover was pronounced for potential glycolate-utilizing bacteria but more subtle for the total bacterial community (Fig. 5.2). Permutational multivariate analysis of variance of the composition of putative glycolate-utilizing bacteria indicates that the assemblage changed substantially over time (PERMANOVA R^2=0.437, p<0.001), and was not significantly affected by depth (PERMANOVA R^2=0.072, p=0.176). Inlet *glcD* assemblages were characterized by comparatively low richness and dominated by OTU 2 (Fig. 5.3), which was abundant in the Emerald Lake assemblage in snow-melt and initial ice-off samples, decreased through time, and was not detected on the final sampling dates. Consequently, the inlet *glcD* assemblages, which were collected in late August and early September, most closely resembled early season (June) assemblages from within Emerald Lake. This relationship between inlet and early-season assemblages was also observed in the total bacterial assemblage (Fig. 5.2).

Single variables highly correlated with temporal changes in *glcD* T-RFLP fingerprints included water residence time, dissolved nitrate+nitrite, total dissolved nitrogen, fluorescence index (indicator of the relative contribution of terrestrial- and lake-derived organic matter (McKnight et al., 2001)), and the ratio of dissolved inorganic nitrogen to total phosphorus (DIN:TP) (Table 5.1). The combination of chemical variables best able to explain observed

patterns in *glcD* T-RFLP fragments was fluorescence index and dissolved inorganic nitrate+nitrite. Correlations were also detected between environmental factors and the relative abundance of individual OTUs for samples collected at a depth of 2 m in Emerald Lake (Fig. 5.4). Residence time was highly correlated with other environmental variables including dissolved inorganic nitrate+nitrite, chlorophyll *a*, and fluorescence index, as well as relative abundance of specific *glcD* restriction fragments.

DISCUSSION

Taxonomic diversity of glcD genes

Similar to *glcD* genes previously described in humic lakes (Paver & Kent, 2010), most OTUs in Emerald Lake belonged to the class *Betaproteobacteria* and fell predominately within two major clades: sequences closely related to *Polynucleobacter* (within 77% amino acid identity) and sequences forming a sister group to *Bordetella* and *Achromobacter* (Paver & Kent, 2010). One difference between *glcD* genes described in humic lakes and those in Emerald Lake is the detection of sequences classified within the *Planctomycetes* in Emerald Lake, which were more abundant in early season samples, comprising roughly 17% of the sequences from early July and 4% in September. Accordingly, *Planctomycetes* have been described in 16S rRNA gene clone libraries from Emerald Lake (Nelson, 2009) but not from the humic lakes (Newton*, et al.*, 2006). *Planctomycetes*-related *glcD* sequences that share 45% amino acid identity with *Planctomycetes glcD* sequences in the current study have previously been detected in *glcD* from surveys of marine systems (Lau*, et al.*, 2007).

Positive associations between phytoplankton or their exudates and organisms within both *Betaproteobacteria* and *Planctomycetes* groups have been described in previous studies. Algal

lysate addition to mesocosms with alpine lake bacterial communities resulted in an enrichment of *Betaproteobacteria*, especially organisms within the betI lineage (Perez & Sommaruga, 2006, Newton*, et al.*, 2011). Additionally, correlations have been detected between population dynamics of phytoplankton and *Betaproteobacteria* within the betII lineage, which contains *Polynucleobacter*, and betIII lineage, which contains *Achromobacter* and *Bordetella*, (Newton*, et al.*, 2006, Newton*, et al.*, 2011) as well as *Polynucleobacter necessarius* subcluster B (Wu & Hahn, 2006). *Planctomycetes* have been detected in 16S rRNA gene libraries from epibacterial communities living on marine macroalgae (Lachnit*, et al.*, 2011), as well as isolated from marine macroalgae (Fukunaga*, et al.*, 2009, Lage & Bondoso, 2011).

Temporal patterns of glcD genes

Temporal patterns were observed in the detection and composition of *glcD* genes. No amplification of *glcD* was detected in samples from Emerald Lake collected prior to ice-off or for three weeks following ice-off for the inlet. Prior to ice off, chlorophyll *a* levels in Emerald Lake were relatively low (0.01 µg/L) compared to the ice-free period (0.11-1.25 µg/L). Low chlorophyll *a* values are indicative of low phytoplankton abundance (Labaugh, 1995), suggesting that autochthonous resources, including glycolate, may not have been readily available prior to ice-off. Chlorophyll *a* concentration and glycolate levels have been significantly positively correlated in regions of the Atlantic Ocean (Leboulanger*, et al.*, 1997). Moreover, during this early-season period (6-20 June) the majority of dissolved organic matter in Emerald Lake was derived from terrigenous sources (Nelson, 2009, Sadro*, et al.*, 2011). This observation suggests that bacterial populations with genetic potential to use glycolate, inferred through *glcD* genes,

specialize on autochthonous resources, as they do not appear to persist on terrestrial-derived organic matter when these autochthonous resources are scarce.

The composition of *glcD* genes exhibited a pronounced temporal trajectory from snowmelt through ice-off and fall turnover. Temporal patterns were similar to those observed in the total bacterial community of Emerald Lake (Nelson, 2009), with a relatively chronological progression from snowmelt to ice-off through fall turnover. Similar successional patterns in putative glycolate-utilizing bacterial assemblages have been previously been observed in north temperate humic lakes (Paver & Kent, 2010). Abundance of *glcD* gene phylotypes, determined by qPCR, have also been shown to change over the development of a marine phytoplankton bloom, with certain phylotypes increasing in abundance while others decreased or remained constant (Lau*, et al.*, 2007).

The temporal trajectory in the putative glycolate-utilizing assemblage was driven by a shift in dominance between OTUs within the *Betaproteobacteria.* Notably, OTU 2, which was similar to the *glcD* sequences from *Achromobacter* and *Bordetella*, dominated early-season samples from Emerald Lake and also dominated samples from the inlet. One possible explanation is that the inlet provides a source of organisms able to use glycolate following snowmelt when run-off from the inlet is high and water residence time in the lake is short, and that there is a growing distinction between the pelagic putative glycolate-utilizing assemblage and those observed in the inlet as the season progressed and water residence time increased. The contribution of bacterial cells imported via inlets to bacterial community composition has previously been demonstrated to be greater in a lake with short water residence time relative to a lake with longer water residence time (Lindström & Bergstrom, 2004). Alternatively, OTU 2, which was negatively correlated with fluorescence index and thus the development of the algal

resource base, may represent an early successional glycolate-utilizing taxon that is the first to appear when conditions for glycolate utilization become favorable.

In addition to short water residence times indicating a substantial influx of bacteria from the landscape (Lindström & Bergstrom, 2004), residence time provides a useful proxy for changes in lake chemistry due to strong correlations observed with nutrients, chlorophyll *a*, and dissolved organic matter source (as measured by the fluorescence index). Accordingly, changes in the composition of the putative glycolate-utilizing bacterial assemblage in Emerald Lake, as well as the total bacterial community described by Nelson (2009), were highly correlated with water residence time. To better understand the mechanisms underlying shifts in the putative glycolate-utilizing assemblage, we determined that the chemical variables most strongly correlated with changes in the *glcD* gene composition were fluorescence index and dissolved inorganic nitrate+nitrite. Fluorescence index and dissolved inorganic nitrate+nitrite were additionally correlated with specific *glcD* OTUs as determined using local similarity analysis as well as the total bacterial community for samples collected during the same time period in 2005 (BIO-ENV ρ=0.709 and ρ=0.724, respectively) (Nelson, 2009). Correspondence between chemical variables correlating with glycolate utilizers and total bacteria may result from the contribution of the glycolate-utilizing component of the bacteria to temporal patterns observed in the total bacteria.

The potential importance of changes in DOM characteristics as a driver of the composition of the glycolate-utilizing assemblage is highlighted by the correlation with fluorescence index, dissolved nitrogen compounds, and the ratio of dissolved inorganic nitrogen to total phosphorus. Fluorescence index is directly related to the composition of DOM, as it is an indicator of the relative proportion of DOM originating from within the lake vs. terrestrial

sources (McKnight*, et al.*, 1993). Although a substantial fraction of lake bacterial production can be supported by terrigenous inputs, lake-derived carbon tends to be more labile and has been shown to be preferred by certain bacterioplankton assemblages (Kritzberg*, et al.*, 2006). In Emerald Lake, it has been demonstrated that daily production of DOM corresponding with periods of photosynthesis (e.g. through release of phytoplankton exudates) is utilized by bacterioplankton within hours of production (Sadro*, et al.*, 2011b). In addition to being labile and rapidly utilized, the composition and abundance of algal-derived DOM is variable. Nutrient concentrations and ratios affect both the composition of the phytoplankton community (Lafrancois*, et al.*, 2003) and the quantity and composition of exudates released by phytoplankton (Hama & Honjo, 1987, Puddu*, et al.*, 2003, Parker & Armbrust, 2005). The composition of algal-derived dissolved organic matter has also been observed to change seasonally (Morris & Skea, 1978). While glycolate represents only one of the potential components of DOM, the results here and in prior studies (Lau & Armbrust, 2006, Lau*, et al.*, 2007, Paver & Kent, 2010) suggest that *glcD* may be a useful functional marker to represent bacteria that respond to algal DOM, and the dynamics of these potential glycolate oxidizers may reflect the response of the heterotrophic bacterial community to the evolving resource base represented by the variable inputs of algal-derived DOM (including glycolate).

Conclusions

This study presents evidence that the composition of the glycolate-utilizing bacterial assemblage is dependent on dissolved organic matter source and composition. As a group, bacteria with the genetic potential to use glycolate appear to specialize on autochthonous resources, resulting in abundances of *glcD* genes below the level of detection when dissolved

organic matter is dominated by terrestrial inputs and within-lake primary production is low. The seasonal progression of putative glycolate-utilizing bacterial assemblage composition corresponds to variation in environmental factors that have been linked to changes in dissolved organic matter characteristics (e.g. fluorescence index, dissolved nitrogen). The strong correlation between the total bacteria and putative glycolate-utilizing bacteria indicate that bacteria able to utilize algal-derived resources may drive bacterial community dynamics during periods when autochthonous dissolved organic matter is available. These results strengthen the evidence for shifts in dissolved organic matter structuring bacterial communities, and highlight the contribution of bacteria specializing on particular suites of resources.

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TABLES

Table 5.1. Correlations between the *glcD* T-RFLP Bray-Curtis similarity matrix and Euclidean distance matrices of environmental variables determined by the BEST algorithm.BV-STEP forward selection was used to select the most strongly correlated combination of chemical variables ($p<0.001$).

Environmental variables	ρ
Matrix generated from combination of chemical variables	
Fluorescence index, dissolved nitrate+nitrite	0.746
Matrices generated from individual variables	
Residence time	0.883
Dissolved nitrate+nitrite	0.710
Total dissolved nitrogen	0.693
Fluorescence index	0.650
DIN:TP	0.639
Schmidt stability	0.552
Buoyancy Frequency	0.517

Abbreviation: DIN:TP, ratio of dissolved nitrate+nitrite to total phosphorus

FIGURES

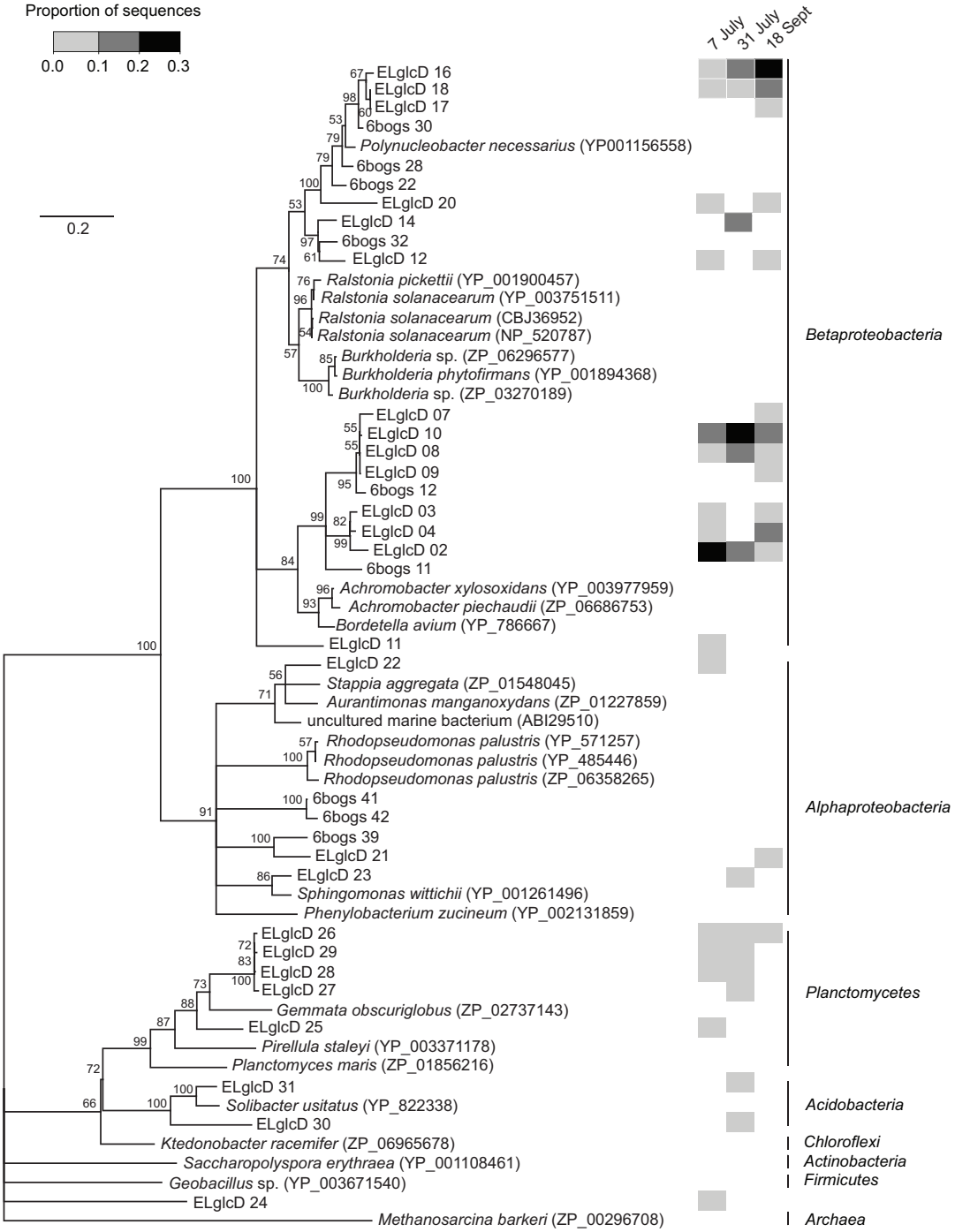

Figure 5.1. Maximum-likelihood tree of *glcD* amino acid sequences from the current study, a survey of humic lakes, and selected sequences from GenBank.OTUs were determined at the 99% DNA similarity level by DOTUR. A heatmap indicates the proportion of sequences an OTU makes up in each clone library out of 41, 70, and 76 sequenced clones from 7 July, 31 July, and 18 September, respectively.The *glcD* sequence from *Methanosarcina barkeri* strain fusaro, an archaeon, was designated as the outgroup to root the tree.Numbers on tree branch nodes are bootstrap values based on 100 replications (only values >50 are shown).Scale bar indicates 2 observed changes per 10 amino acids.

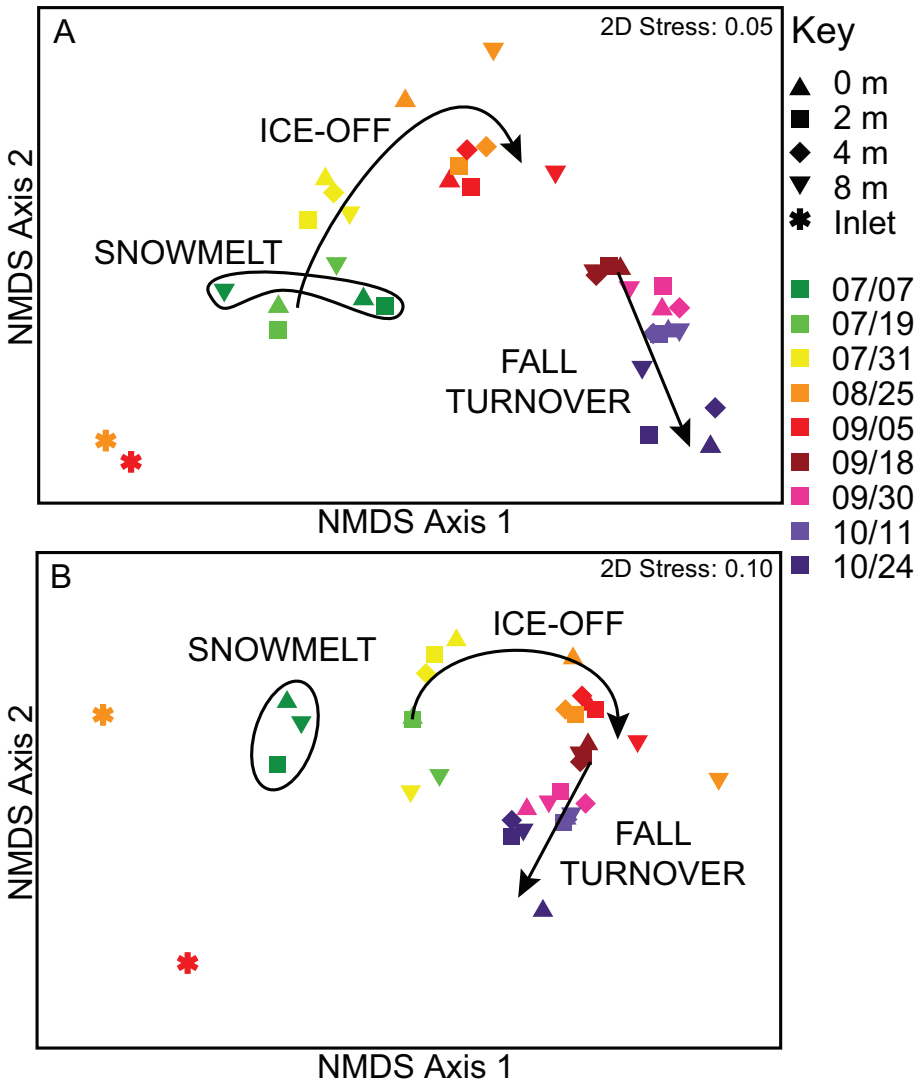

Figure 5.2. Non-metric multidimensional scaling ordination of glycolate-utilizing bacterial assemblages in Emerald Lake assessed by *glcD* T-RFLP (A) and bacterial communities characterized by Nelson (2009) using 16S rRNA T-RFLP (B).Symbol color represents date of sample collection in 2005 and shape represents the location of sample collection (i.e. depth within Emerald Lake or samples collected from the inlet). No *glcD* amplicons were produced from samples collected in June (pre-ice off).

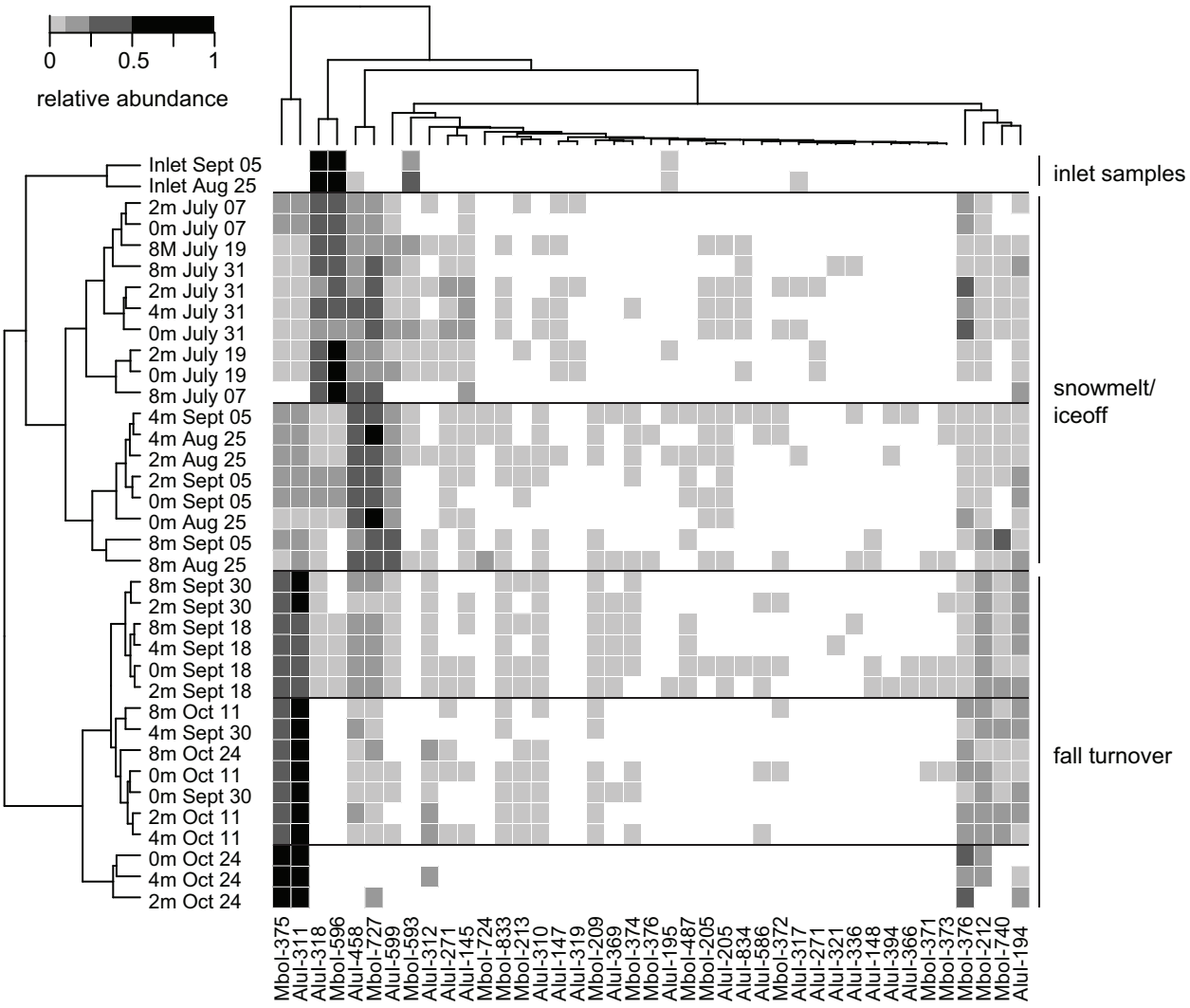

Figure 5.3. Relative abundance of terminal restriction fragments resulting from digests of the *glcD* gene with *AluI* or *MboI* restriction in each sample. Dendrograms represent complete linkage hierarchical clustering of samples and restriction fragments. The two dominant pairs of restriction fragments, MboI_375/AluI_311 and MboI_596/AluI_318, correspond to OTUs 16 and 2, respectively.

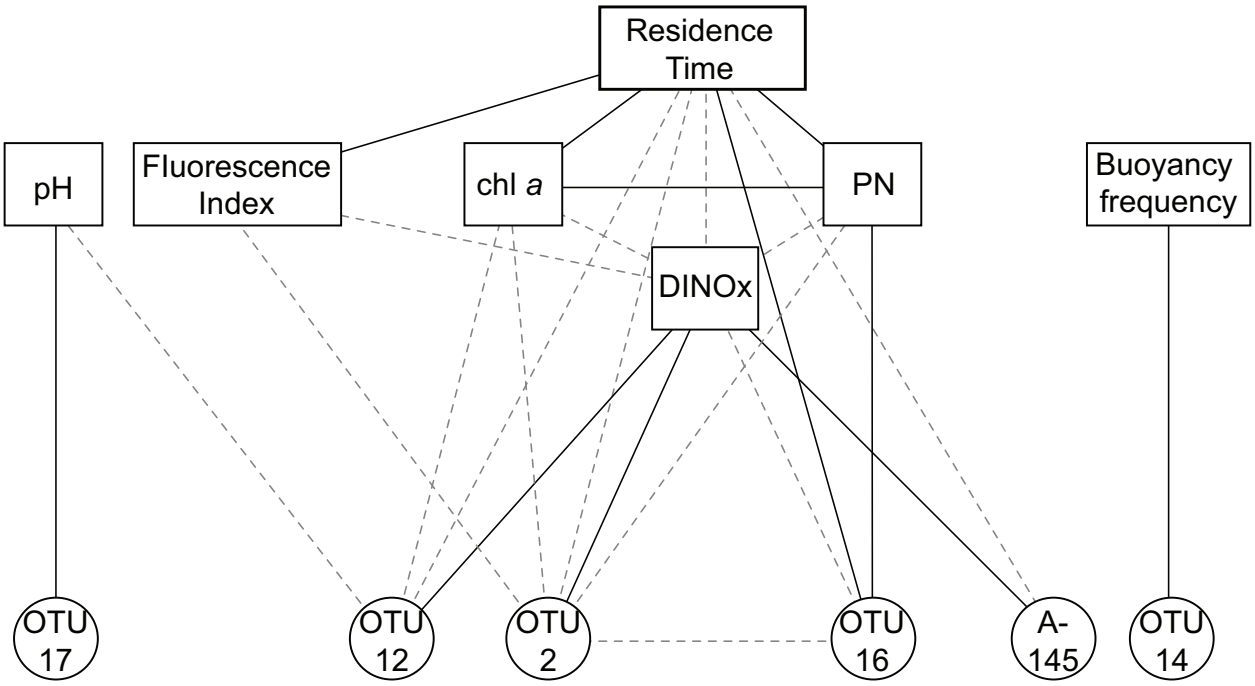

Figure 5.4. Correlations between *glcD* terminal restriction fragments and environmental parameters at 2 m depth in Emerald Lake detected using local similarity analysis. Black lines indicate a positive correlation between two variables while dotted grey lines indicate a negative correlation between two variables. Terminal restriction fragment labels indicate the corresponding OTU as identified from *in silico* digests of *glcD* sequences, with the exception of A-145 (145 bp T-RF observed in the *AluI* digest), which was not predicted for any sequences. Abbreviations: DINOx, dissolved inorganic nitrate+nitrite; PN, particulate nitrogen; chl *a*, chlorophyll *a.*

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

Phytoplankton affect the composition and seasonal succession of bacterial communities and *Polynucleobacter* subtypes. Community- and assemblage-level effects result from interactions between specific phytoplankton and bacterial populations (Fig. 6.1). Moreover, the effects of phytoplankton change under different temperature and light conditions, making interactions between phytoplankton and bacteria vary over depth and time. The observation that phytoplankton succession effects changes in bacterial community composition raises a number of questions. Results presented in this dissertation provide insight into some of these questions while others are left open as areas for further study.

What mechanisms contribute to the influence of phytoplankton on bacteria?

Based on environmental time-series observations and manipulative experiments, inferences can be made about how phytoplankton interact with bacteria. As described in Chapter 2, interactions with phytoplankton appear to be primarily positive due to phytoplankton species enriching for certain members of the bacterial community. However, this result is based on observations at surface conditions – warm temperature, and high light – and may not describe interactions throughout the water column. The two mechanisms of phytoplankton influence with the most support based on the presented data are exudate release and serving as a habitat.

Phytoplankton release a species-specific combination of exudates (e.g., sugars, amino acids, sugar-alcohols) that serves as an evolving resource base to bacteria over the course of phytoplankton seasonal succession (Hellebust, 1965; Fogg, 1983; Sommer et al., 2012). Exudates from different species of phytoplankton and macroalgae have been shown to induce

changes in bacterial community composition in marine mesocosm experiments (Sarmento and Gasol, 2012; Nelson et al., 2013). The glycolate oxidase subunit D (*glcD*) gene indicates genetic potential to utilize the algal exudate glycolate and can be characterized to study a subset of bacteria able to use exudates in the environment (Lau and Armbrust, 2006; Lau et al., 2007; Paver and Kent, 2010). In the humic lakes studied in Chapters 2-4, changes in phytoplankton population abundances explained an average of 39% of temporal variation in *glcD* genes, and certain *glcD* OTUs were correlated with specific phytoplankton populations (Paver and Kent, 2010). In Chapter 5, *glcD* genes were not detected in Emerald Lake during ice-cover when phytoplankton resources were scarce, but exhibited significant temporal patterns when resources were available. The availability of algal exudates therefore appears to affect the abundance and development of bacteria with genetic potential to use glycolate, providing support for exudates as a mechanism of phytoplankton influence on bacterial community composition. Results from the phytoplankton, temperature, and light manipulation study presented in Chapter 4 are consistent with the influence of exudates. The effect of temperature was greater for bacteria incubated with phytoplankton from the "away" lake than from their "home" lake. Temperature appears to amplify the novel effects of "away" phytoplankton, possibly due to the presence of unfamiliar exudates. Light availability affects the abundance and composition of algal exudates (Parker and Armbrust, 2005; Panzenbock, 2007), which may explain differences in bacterial community composition in "away" phytoplankton treatments following incubation at high and low light at the warmest temperature.

Results from multiple investigations provide evidence for the effects of phytoplankton association on bacterial community composition. In the algal exchange experiment presented in Chapter 2, putative phytoplankton-associated and phytoplankton-colonizing bacteria that were

enriched in phytoplankton treatments explained a combined average of 11.8% of the difference between phytoplankton treatments and no-phytoplankton controls. Additionally, the particle-associated ARISA bacterial OTU 684 (Paver, 2009) was consistently correlated with *Gymnodinium fuscum* in environmental time-series observations and increased in abundance when incubated with phytoplankton assemblages containing *G. fuscum* in the algal exchange experiment presented in Chapter 2. Based on 16S rRNA gene sequencing, OTU 684 is an uncultured *Deltaproteobacteria*. Further investigation of the relationship between OTU 684 and *G. fuscum* will enhance the understanding of bacterial diversity and how different phytoplankton and bacteria interact. A subset of *Polynucleobacter* subtypes also appears to have a phytoplankton-associated lifestyle based on the pre-incubation differences in *Polynucleobacter* composition due to phytoplankton treatment described in Chapter 3. Finally, enrichment of bacterial *Chlamydiae* OTUs in experimental phytoplankton treatments presented in Chapters 2 and 4 suggests that phytoplankton serve as hosts to intracellular organisms.

Which bacterial taxa benefit from interactions with phytoplankton?

In the algal exchange experiments conducted for Chapters 2 and 4, phytoplankton enriched for OTUs from a wide range of phyla, including the *Alpha-*, *Beta-*, *Gamma-*, and *Delta-proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chlamydiales*, *Firmicutes*, *Fusobacteria*, and *Verrucomicrobia*. OTUs identified to the betaproteobacterial betI-A clade (*Limnohabitans* genus) were enriched in both algal-exchange experiments. Bacteria in the betI-A clade have been observed in algal cultures (Šimek et al., 2011) and co-occurring with periods of high extracellular phytoplankton production in a dam reservoir (Šimek et al., 2008). These organisms have the ability to respond quickly to changes in environmental conditions due to

relatively short population turnover times (Šimek et al., 2001; Šimek et al., 2005; Šimek et al., 2006; Alonso et al., 2009). OTUs from the alphaproteobacterial alfI clade, related to the *Rhizobiales*, were also enriched in both experiments. Information on the ecology of alfI bacteria in freshwater is lacking (Newton et al., 2011); however, *Rhizobiales* is known to be a heterogeneous order that includes pathogens as well as plant-associated nitrogen-fixing bacteria (Kuykendall et al., 2005). Chapter 3 demonstrated that phytoplankton enrich for a subset of subtypes in the betaproteobacterial *Polynucleobacter* genus. In the temperature and light manipulation mesocosm experiment presented in Chapter 4, an OTU from the *Polynucleobacter* PnecA tribe was enriched when bacteria were incubated with TB phytoplankton. *Polynucleobacter* is a cosmopolitan and ubiquitous freshwater bacterial genus that can exceed 60% of bacterial cells in certain lakes (Hahn et al., 2005; Wu and Hahn, 2006a; Wu and Hahn, 2006b; Jezberová et al., 2010; Newton et al., 2011). Interactions between phytoplankton and *Polynucleobacter* had previously been inferred from temporal patterns and depth distribution of PnecB in Lake Mondsee (Wu and Hahn, 2006b) and a survey of bacteria with a gene for the utilization of the algal exudate glycolate (Paver and Kent, 2010).

How does the effect of phytoplankton change under different environmental conditions?

The effect of phytoplankton on bacterial community composition depends on environmental conditions. In the phytoplankton, temperature, and light manipulation experiment presented in Chapter 4, temperature generally amplified the effect of phytoplankton on aquatic bacterial community composition. As temperature increased, the pattern of change depended on the light level, suggesting that light modifies the interaction between phytoplankton and bacteria. For certain OTUs, temperature and, to a lesser extent, light conditions determined whether they

were enriched or depleted in a given phytoplankton treatment. A *Chlamydiales* OTU was enriched by TB phytoplankton only in mesocosms that were incubated at the warmest temperature and was particularly abundant at high light levels.

The effect of nutrient availability on phytoplankton interactions with bacteria is not covered in this dissertation, but that is potentially highly relevant to understanding the effect of phytoplankton on bacterial community dynamics. Phosphorus limitation increases bactivory in algal flagellates (Nygaard and Tobiesen, 1993) and changes the quality of exudates and their effect on bacterial community composition (Puddu et al., 2003). Nitrogen source (nitrate vs. ammonia), light, and temperature have synergistic effects on exudate production in the marine centric diatom *Thalassiosira pseudonana* (Parker and Armbrust, 2005).

How do ecological drivers combine to determine bacterial seasonal successional patterns?

Biotic interactions, environmental factors, and stochastic processes act together to affect bacterial community composition. The observations made in Chapters 2 and 3 that specific pairs of correlated phytoplankton and bacterial OTUs were rarely detected in multiple time series suggest that the effect of phytoplankton depends on the effects of other drivers of bacterial community composition. Grazing pressure may affect bacterial response to phytoplankton. In Crystal Bog, one of the humic lakes studied, an annual early summer increase in the abundance of mixotrophic phytoplankton and heterotrophic nanoflagellates co-occurs with a dramatic drop in bacterial richness and abundance (Kent et al., 2004). In Chapter 4, temperature, light, and the interaction between temperature and light were shown to have the potential to influence bacterial communities directly and indirectly by affecting interactions between phytoplankton and bacteria. Further characterization of how biotic and abiotic drivers act in concert to effect

changes in bacterial community composition is needed in addition to studies that use experimental findings to interpret patterns of change observed in the environment.

Toward a predictive framework for lake bacterial community composition

The effects of phytoplankton, light, and temperature described in this dissertation contribute to a growing body of knowledge of factors that affect bacterial community temporal patterns and the abundance of specific bacterial populations within a community. Determination of axes that define niches and functional trait-based classification of bacterial taxa will facilitate integration of this information into a generalizable predictive framework for bacterial community dynamics. Lab measured functional traits have shown promise for predicting phytoplankton response to resource availability in the environment (Litchman et al., 2010; Edwards et al., 2013). For phytoplankton, traits with consequences for fitness include those relating to light and nutrient utilization, grazer and parasite susceptibility, morphology, temperature optima, and reproductive strategy (Litchman and Klausmeier, 2008). Many of these same traits are likely to affect the fitness of bacteria (Pernthaler, 2005; Jones and Lennon, 2010; Martinez-Garcia et al., 2012; Overmann, 2013). Fitness of certain populations of bacteria also likely depends on interactions with other organisms, including phytoplankton. Because many important freshwater bacterial taxa lack cultured representatives (Newton et al., 2011), information about bacterial traits must be inferred from various types of studies, including: 1) observations of relative abundance patterns (or activity) in the environment and how taxa respond to experimental manipulations similar to the ones presented in this dissertation, 2) genomic analyses which can link phylogenetically identified taxa with specific functional genes (Martinez-Garcia et al., 2012), and 3) experiments determining which bacterial taxa take up certain substrates (Sarmento

and Gasol, 2012; Nelson et al., 2013). A predictive trait-based framework of bacterial community dynamics coupled with information about the processes carried out by different bacterial taxa has the potential to enhance our ability to forecast alterations in ecosystem function due to environmental change.

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FIGURES

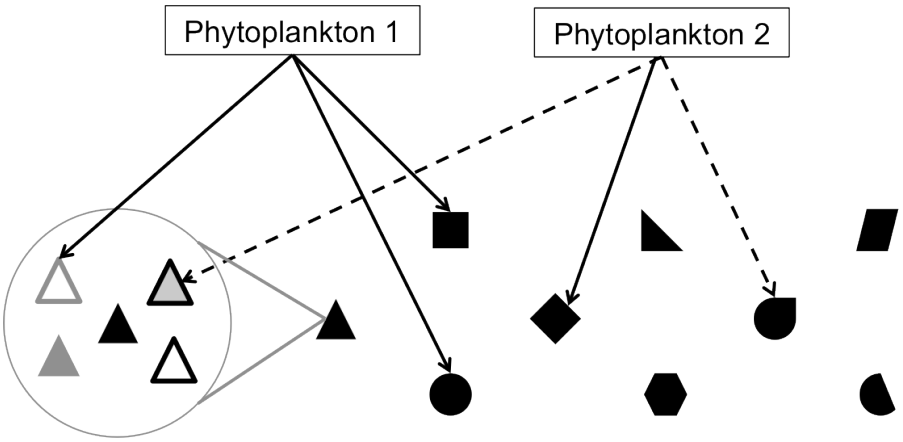

Figure 6.1. Phytoplankton affect bacterial community composition through positive (solid arrows) and negative (dotted arrows) interactions with specific populations of bacteria (black shapes). Additionally, phytoplankton interactions differentially affect microdiverse subtypes of bacteria (shaded triangles) that may be aggregated in into a single operational taxonomic unit depending on the method used for bacterial community characterization.

APPENDIX A

SUPPLEMENTAL MATERIAL FOR CHAPTER 2

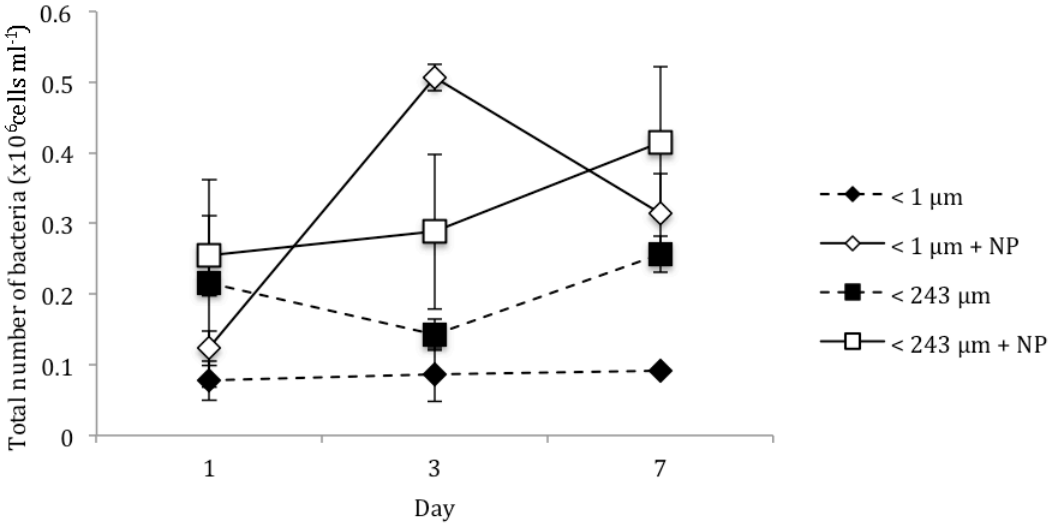

Figure A.1. Total number of bacteria in mesocosms (average ± standard error) with (+NP) and without added nitrogen and phosphorus on days 1, 3, and 7 from experiments described by Kent et al. 2006 (unpublished data). This experiment was conducted in Crystal Bog using the same mesocosm containers and incubation length used in the current study. The <1µm size fraction is analogous to the no-phytoplankton control treatment in this study and <243µm size fraction is similar to phytoplankton assemblage treatments. Without added nutrients, bacterial abundance remains relatively stable over the incubation period.

Table A.1. Initial and final phytoplankton cell counts (cells ml^{-1} ± standard error) over a seven-day pilot experiment. No phytoplankton cells were detected in initial samples collected from no-phytoplankton control treatments.

Phytoplankton	SSB phytoplankton						No phytoplankton		
Bacteria	CB		SSB		TB		CB	SSB	TB
	initial	final	initial	final	Initial	final	final	final	final
Asterionella	$1.29 \pm 0.25 \times 10^4$	$1.29 \pm 0.69 \times 10^4$	$1.93 \pm 0.12 \times 10^4$	$3.57 \pm 0.68 \times 10^4$	$1.14 \pm 0.14 \times 10^4$	$1.51 \pm 0.32 \times 10^5$	n.d.	n.d.	n.d.
Ceratium	$3.28 \pm 3.28 \times 10^4$	n.d.	$2.62 \pm 1.73 \times 10^4$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cryptomonas	$9.68 \pm 2.72 \times 10^3$	$4.01 \pm 3.67 \times 10^3$	$1.11 \pm 0.20 \times 10^4$	$1.77 \pm 0.85 \times 10^4$	$8.26 \pm 2.10 \times 10^3$	$5.43 \pm 2.90 \times 10^3$	n.d.	n.d.	n.d.
Dinobryon	$1.24 \pm 0.16 \times 10^3$	$6.38 \pm 4.00 \times 10^3$	$2.04 \pm 1.02 \times 10^3$	$7.52 \pm 4.49 \times 10^4$	$2.23 \pm 0.77 \times 10^3$	$3.77 \pm 0.47 \times 10^4$	$1.24 \pm 1.24 \times 10^2$	$1.86 \pm 1.86\ 10^2$	n.d.
Euglena	$7.70 \pm 7.70 \times 10^2$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Fragelleria	n.d.	n.d.	n.d.	n.d.	$1.45 \pm 1.45 \times 10^2$	$1.45 \pm 1.45 \times 10^2$	n.d.	n.d.	n.d.
G. Fuscum	$1.73 \pm 0.33 \times 10^5$	$4.03 \pm 4.03 \times 10^3$	$5.24 \pm 1.07 \times 10^4$	$2.42 \pm 0.70 \times 10^4$	$1.69 \pm 0.21 \times 10^5$	$3.22 \pm 2.64 \times 10^4$	n.d.	n.d.	n.d.
Gloecystis	$2.41 \pm 1.39 \times 10^2$	$3.21 \pm 0.80 \times 10^2$	n.d.	n.d.	$4.82 \pm 1.39 \times 10^2$	$8.83 \pm 5.26 \times 10^2$	n.d.	n.d.	n.d.
Mallomonas	n.d.	$1.14 \pm 1.14 \times 10^3$	$7.39 \pm 2.48 \times 10^3$	$9.10 \pm 5.77 \times 10^3$	$1.14 \pm 0.40 \times 10^4$	$1.19 \pm 0.45 \times 10^4$	n.d.	n.d.	n.d.
Oocystis	$1.95 \pm 1.95 \times 10^2$	n.d.	$1.95 \pm 1.95 \times 10^2$	$3.91 \pm 1.95 \times 10^2$	$5.86 \pm 3.38 \times 10^2$	$1.95 \pm 1.95 \times 10^2$	n.d.	n.d.	n.d.
P. cinctum	$7.43 \pm 7.43 \times 10^3$	$7.43 \pm 7.43 \times 10^3$	n.d.	$7.43 \pm 7.43 \times 10^3$	$1.49 \pm 0.74 \times 10^4$	$3.34 \pm 2.73 \times 10^4$	n.d.	n.d.	n.d.
P. limbatum	$1.05 \pm 0.53 \times 10^6$	$6.58 \pm 4.75 \times 10^5$	$1.05 \pm 0.70 \times 10^5$	$3.69 \pm 1.47 \times 10^5$	$1.69 \pm 0.25 \times 10^6$	$9.74 \pm 2.30 \times 10^5$	$2.63 \pm 2.63 \times 10^4$	n.d.	n.d.
P. umbanotum	$2.19 \pm 0.56 \times 10^4$	$1.09 \pm 0.87 \times 10^4$	$9.37 \pm 5.41 \times 10^3$	$9.37 \pm 5.41 \times 10^3$	$7.50 \pm 1.87 \times 10^4$	$1.41 \pm 0.00 \times 10^4$	n.d.	n.d.	n.d.
Synura	$2.94 \pm 1.81 \times 10^5$	$3.46 \pm 1.95 \times 10^5$	$6.92 \pm 3.46 \times 10^4$	$2.77 \pm 0.35 \times 10^5$	$1.21 \pm 0.62 \times 10^5$	$9.00 \pm 1.51 \times 10^5$	n.d.	n.d.	n.d.

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APPENDIX B

SUPPLEMENTAL MATERIAL FOR CHAPTER 3

Table B.1. *Polynucleobacter* T-RFs that significantly increased or decreased over the mesocosm incubation period in phytoplankton treatments relative to no-phytoplankton controls as determined by EDGE analysis and whose abundance was positively (+) or negatively (-) correlated with abundance of a specific phytoplankton species in one or more weekly environmental time series observations of SSB and TB from May-August 2003 and 2008 as determined by local similarity analysis.

	Mesocosm Experiment: Phytoplankton and Bacterial Treatments				Environmental Time Series: Phytoplankton Species												
	SSB phytoplankton		TB phytoplankton														
T-RFs	SSB	TB	SSB	TB	*Asterionella*	*Crucigenia*	*Cyclotella*	*Cryptomonas*	*Dinobryon*	*G. fuscum*	*Mallomonas*	*P. cinctum*	*P. limbatum*	*Pumbumb*	*Scenedesmus*	*Synura*	*UCC*
HR_180		+^										+	+			+	
HR_422		-		-								-					
HR_826			+^	+				-		+							
HF_069			+^					+	-							+	
HF_176				+			+		+								+
HF_223			-*	-*		+											
HF_847			+^	+^											+		
RR_056				+		-					-	-					
RR_434		-		-	-			+	-								
RR_627				+									-				
RF_192		-*	-	-*		+						+	+				
RF_220		+		+										-			
RF_329				-													+
RF_463		-	-*	-						+						+	
RF_633		-*		-	+												
RF_784			+^	+^						+		+					-

^ Consistently phytoplankton-associated

* Consistently phytoplankton-depleted

Abbreviations: SSB, South Sparkling Bog; TB, Trout Bog; *G. fuscum*, *Gymnodinium*; *P. cinctum, P. limbatum* and *P. umbonatum*, *Peridinium*; *P. umb. umb.*, *Peridinium umbonatum umbonatum;* UCC, unflagellated colonial chrysophyte.

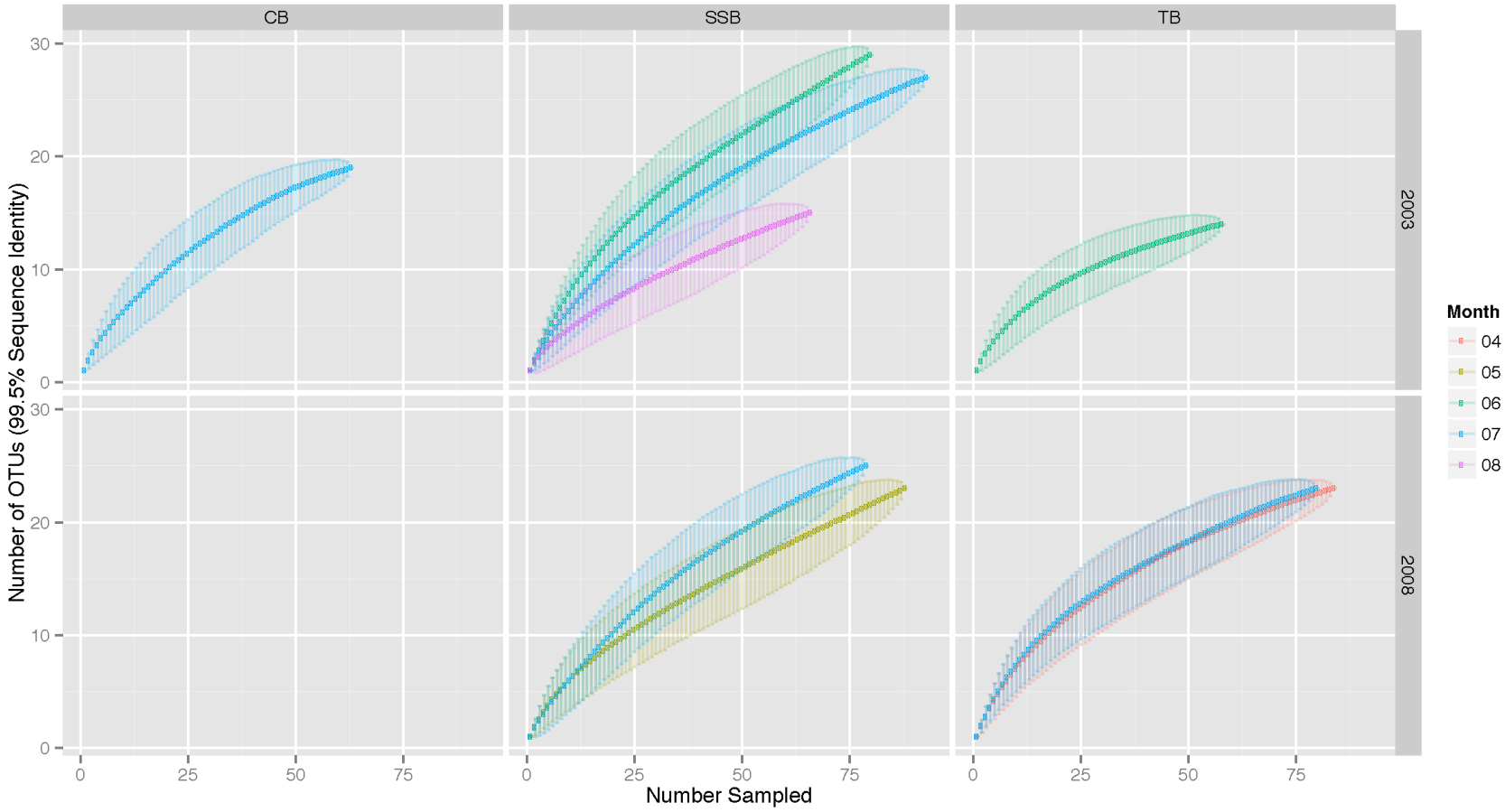

Figure B.1. Rarefaction curves for each clone library. Error bars represent 95% confidence intervals determined from 1000 subsampling iterations.

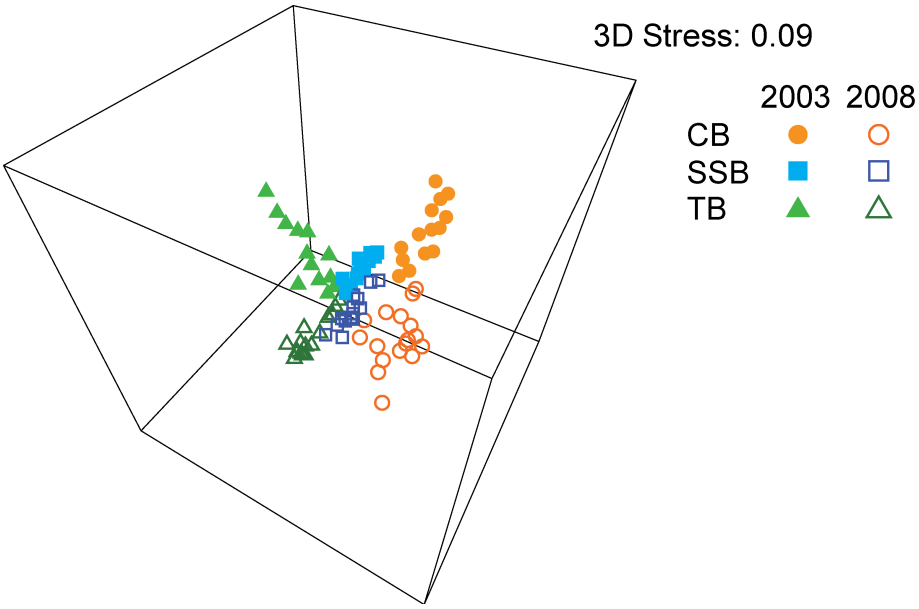

Figure B.2. Non-metric multi-dimensional scaling ordination of *Polynucleobacter* composition characterized by *ccoN* gene T-RFLP analysis for all samples collected from CB, SSB, and TB in 2003 and 2008. We detect differences among lakes (PERMANOVA R^2=0.21, $p<0.001$) and between years (PERMANOVA R^2=0.26, $p<0.001$).

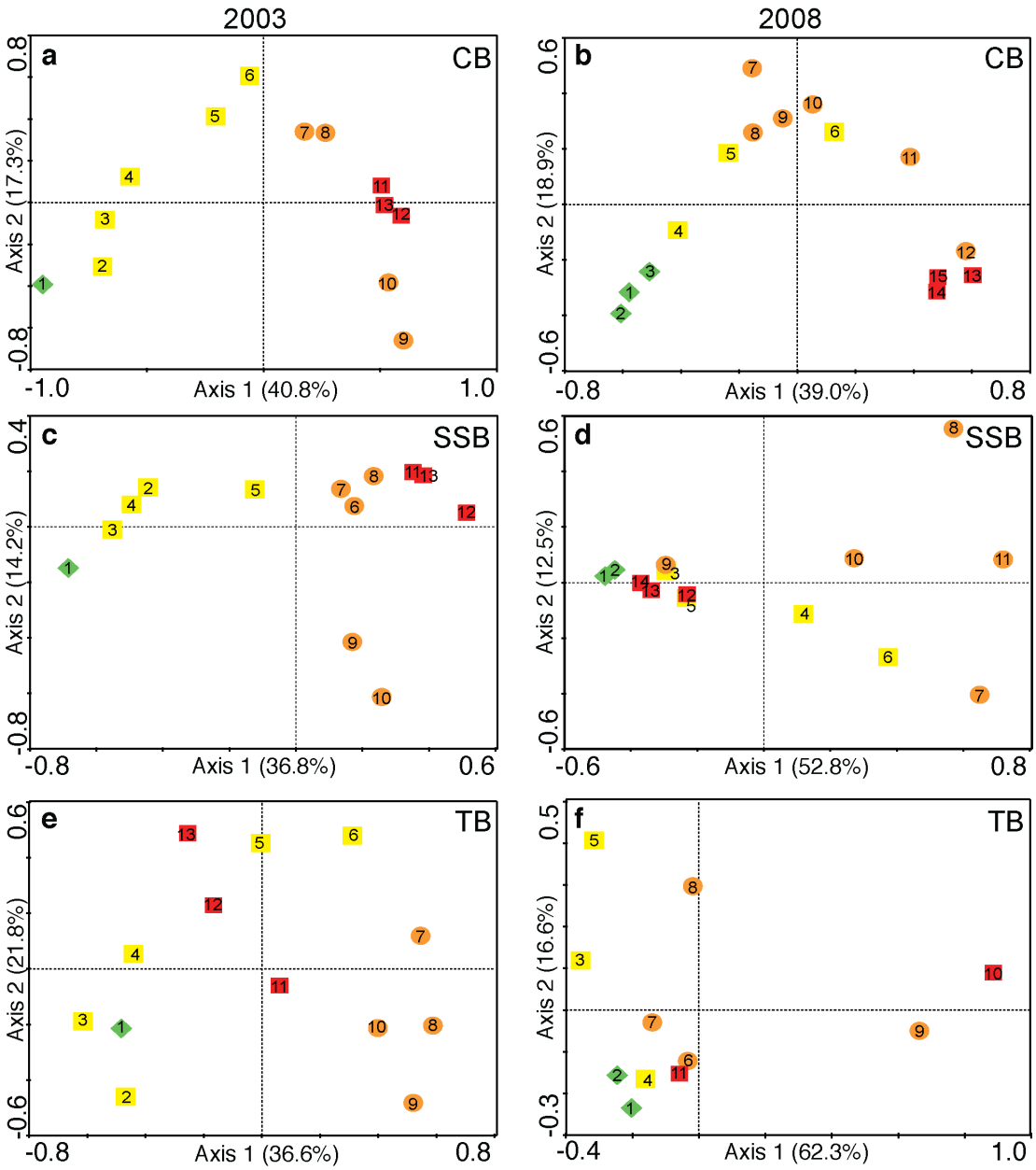

Figure B.3. Correspondence analysis of *Polynucleobacter* assemblages characterized by *ccoN* gene T-RFLP from samples collected weekly from Crystal Bog (a, b), South Sparkling Bog (c, d), and Trout Bog (e, f) in 2003 and 2008. Points represent sample dates and are numbered consecutively with colors and shapes corresponding to the month samples were collected (green diamond, may; yellow square, June; orange circle, July; red square, August). Percent of observed variation explained by each axis is indicated in parentheses. Observed trajectories of change in the composition of *Polynucleobacter* are similar to those observed for the phytoplankton assemblages as described by Kent and colleagues (2007).

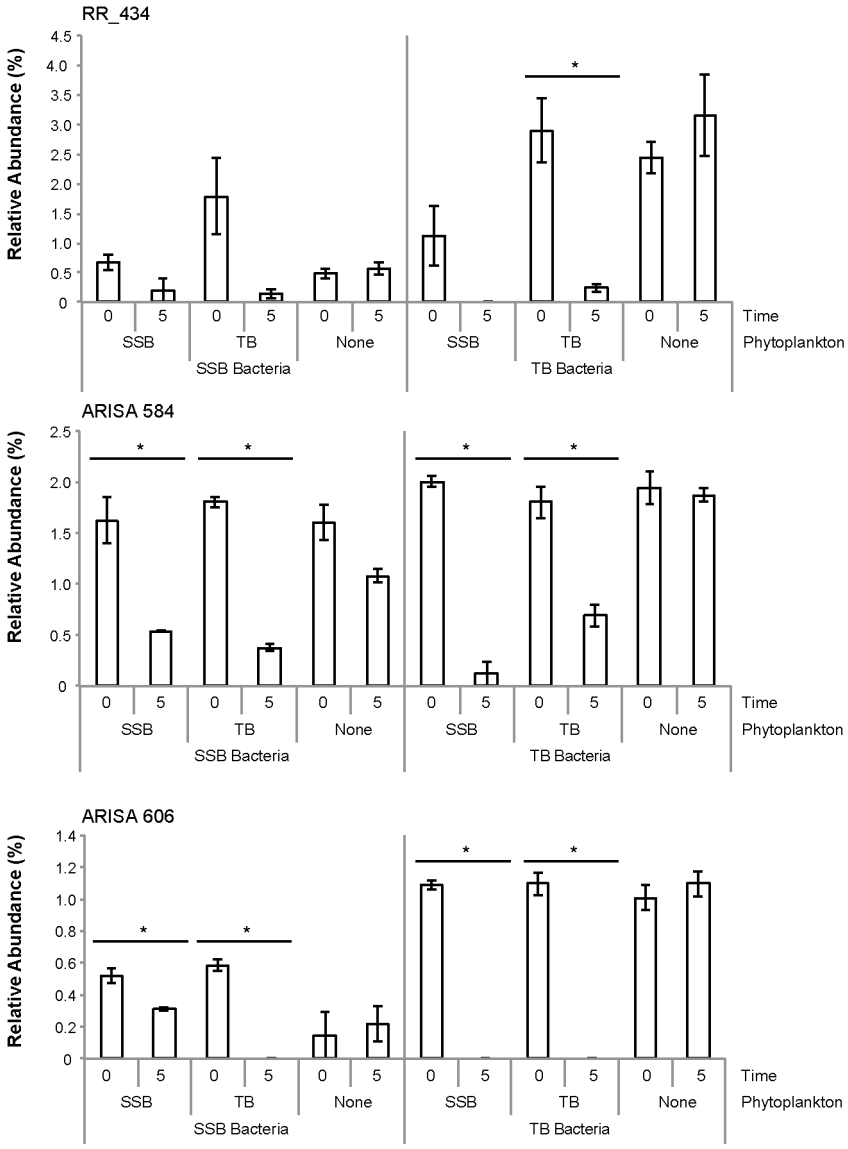

Figure B.4. Relative abundance of *Polynucleobacter* fragment RR_434 and ARISA fragments 584 and 606 before (time 0) and after (time 5) incubation in an experiment combining bacteria from SSB or TB with SSB, TB, or no phytoplankton. Relative abundance that significantly changed from time 0 to time 5 is indicated by *.

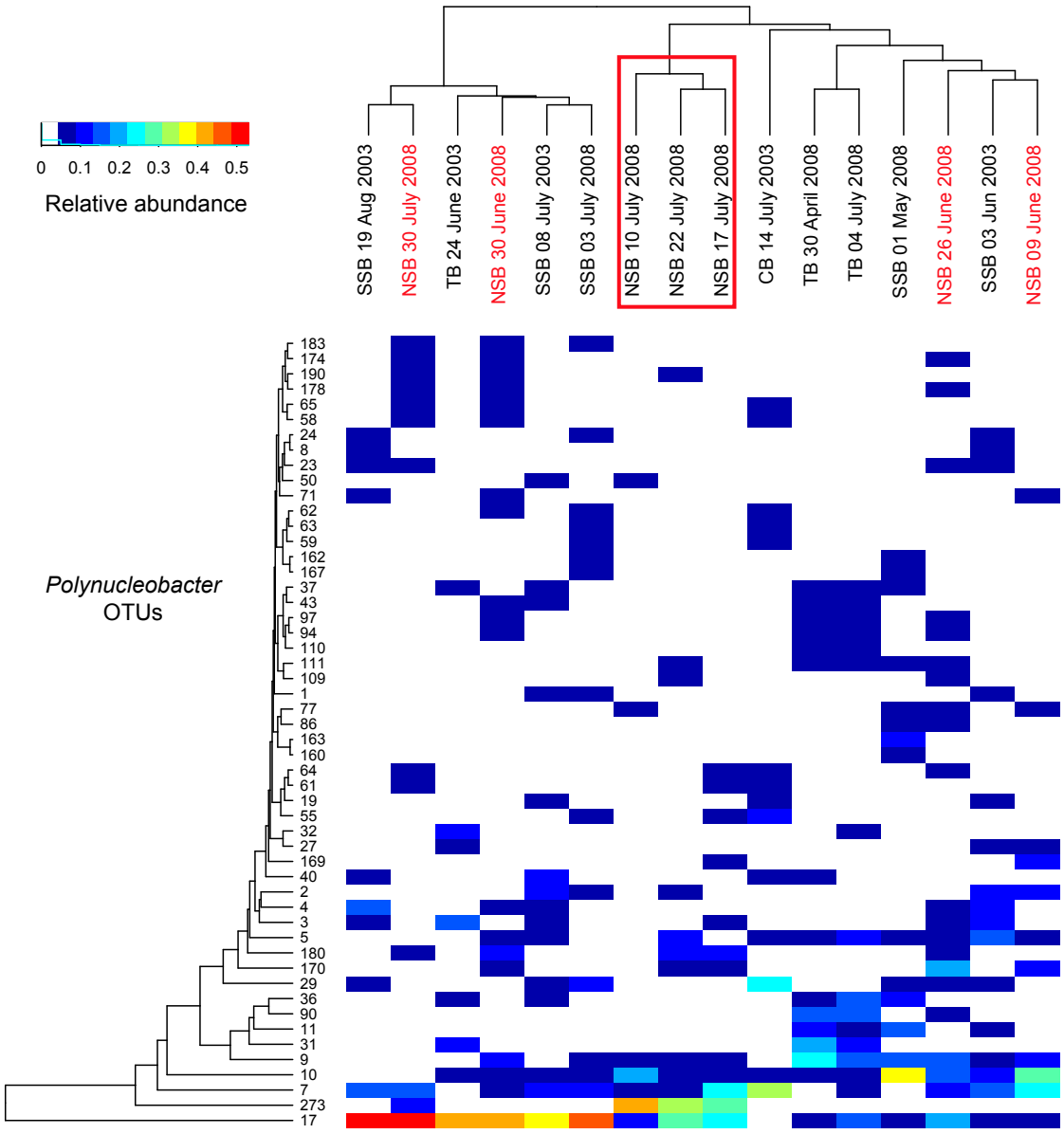

Figure B.5. Relative abundance of each *Polynucleobacter* OTU defined at the >99.5% similarity level by mothur in clone libraries constructed from this study and clone libraries constructed from North Sparkling Bog (NSB). Aside from samples collected within two weeks following an artificial whole-lake mixing of NSB (indicated by the red rectangle), the distribution of *Polynucleobacter* OTUs collected from NSB is similar to our study lakes, especially SSB. Dendrograms represent complete linkage clustering of *Polynucleobacter* OTUs and samples based on Euclidian distance. NSB clone libraries from Youngblut et al. (2013).

REFERENCES

Kent, A.D., Yannarell, A.C., Rusak, J.A., Triplett, E.W., and McMahon, K.D. (2007) Synchrony in aquatic microbial community dynamics. *ISME J* **1**: 38-47.

Youngblut, N.D., Shade, A., Read, J.S., McMahon, K.D., and Whitaker, R.J. (2013) Lineage-specific responses of microbial communities to environmental change. *Appl Environ Microbiol* **79**: 39-47.

APPENDIX C

SUPPLEMENTAL MATERIAL FOR CHAPTER 4

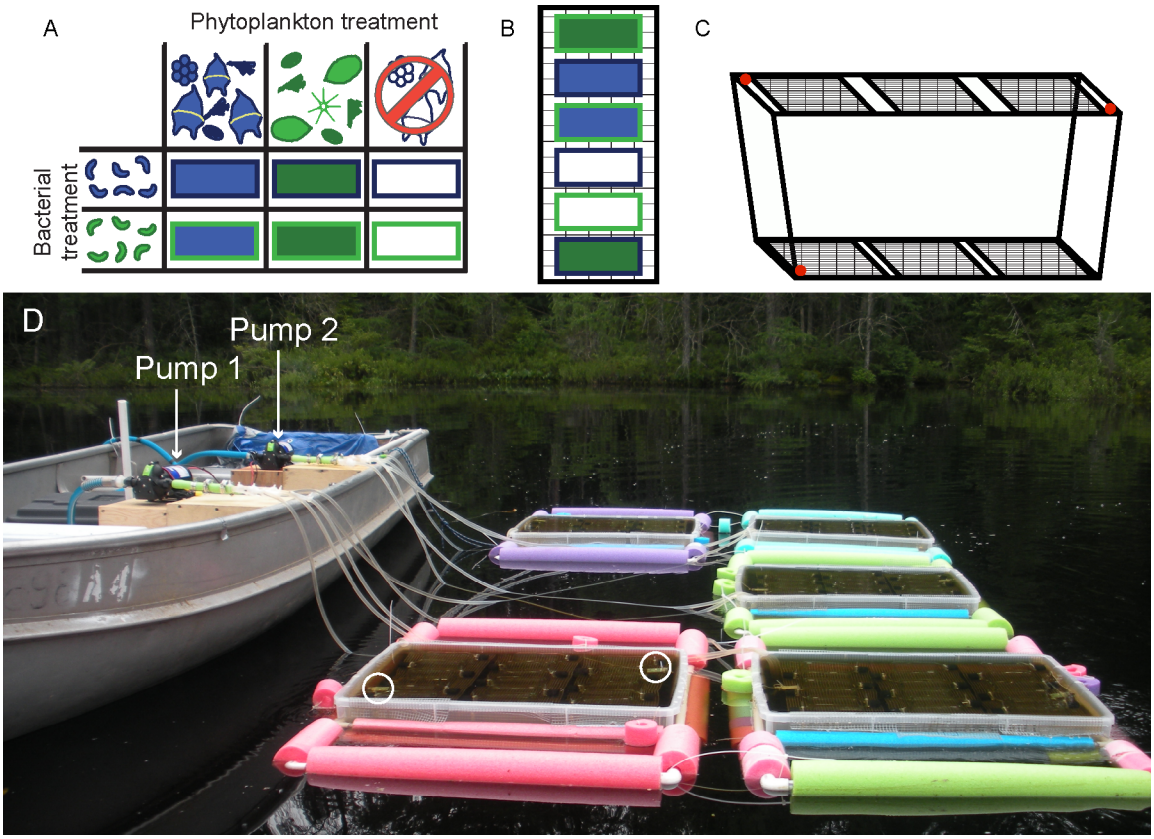

Figure C.1. Experimental design diagram. Bacteria from each lake were combined with phytoplankton from each lake or no phytoplankton as a control (A). One bottle from each combination of bacteria and phytoplankton was fastened to wire mesh supports in a randomized, pre-determined order (B). Three wire mesh blocks were placed on the top and bottom of each of five plastic container incubators (C). Red circles indicated the relative placement of three hobo data loggers used to record temperature and light in each container. Different temperature conditions were maintained in each of the five plastic container incubators by mixing different proportions of warm, surface water from pump 1 and cold, sub-surface water from pump 2 (D). White circles indicate placement of surface hobo data loggers in one incubator.

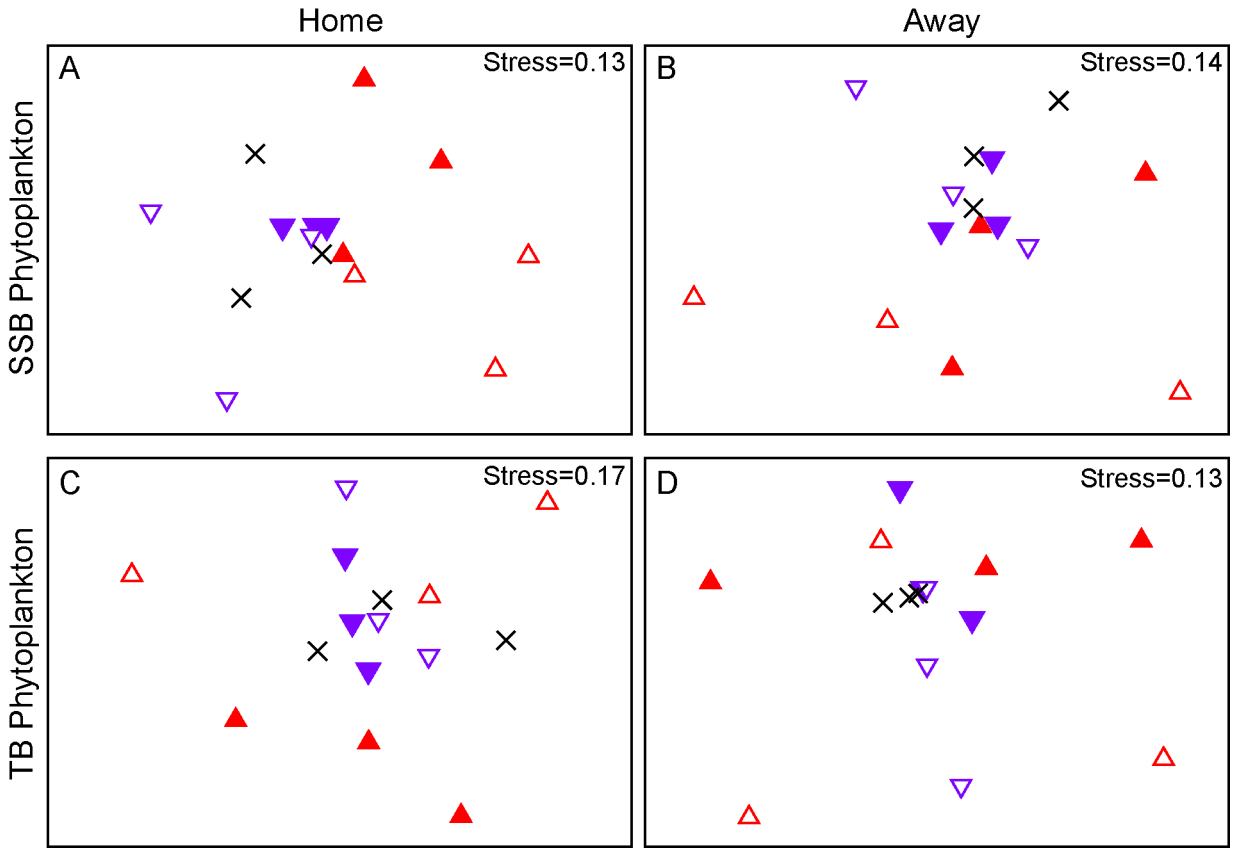

Figure C.2. Non-metric multidimensional scaling ordination of phytoplankton community composition based on Bray-Curtis similarity of 23S rRNA plastid OTU relative abundances in SSB phytoplankton home (A) and away (B) and TB phytoplankton home (C) and away (D) mesocosms. Symbols correspond to initial community composition (X), and post-incubation community composition in mesocosms incubated at the warmest (red) and coldest (purple) temperatures at high (open) and low (solid) light levels.

The following figures provide a description of results from 23S plastid pyrosequencing:

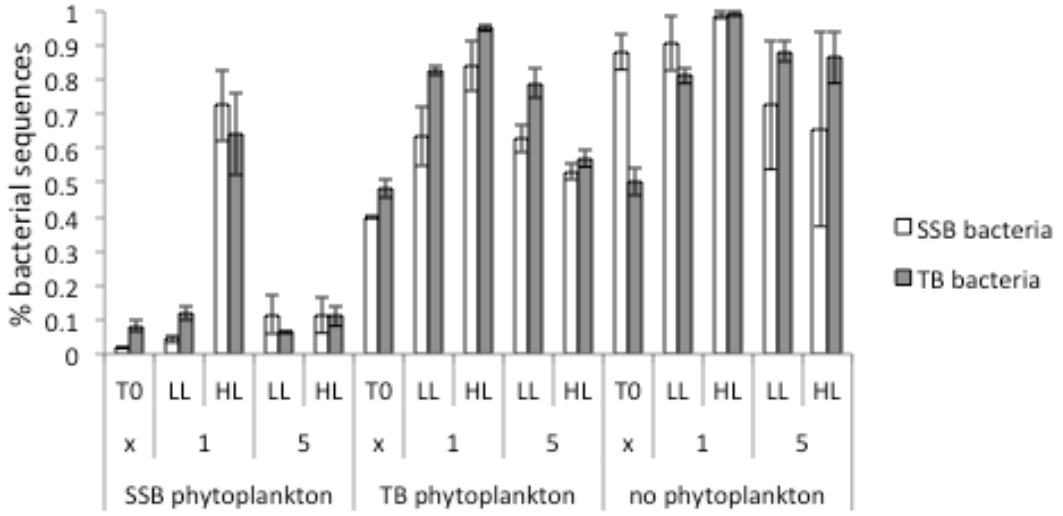

Figure C.3. Percent bacterial sequences recovered from 23S rRNA plastid pyrosequencing of each treatment (average ± standard error). Efficiency of 23S rRNA plastid pyrosequencing was greater for SSB phytoplankton treatments than for TB phytoplankton treatments.

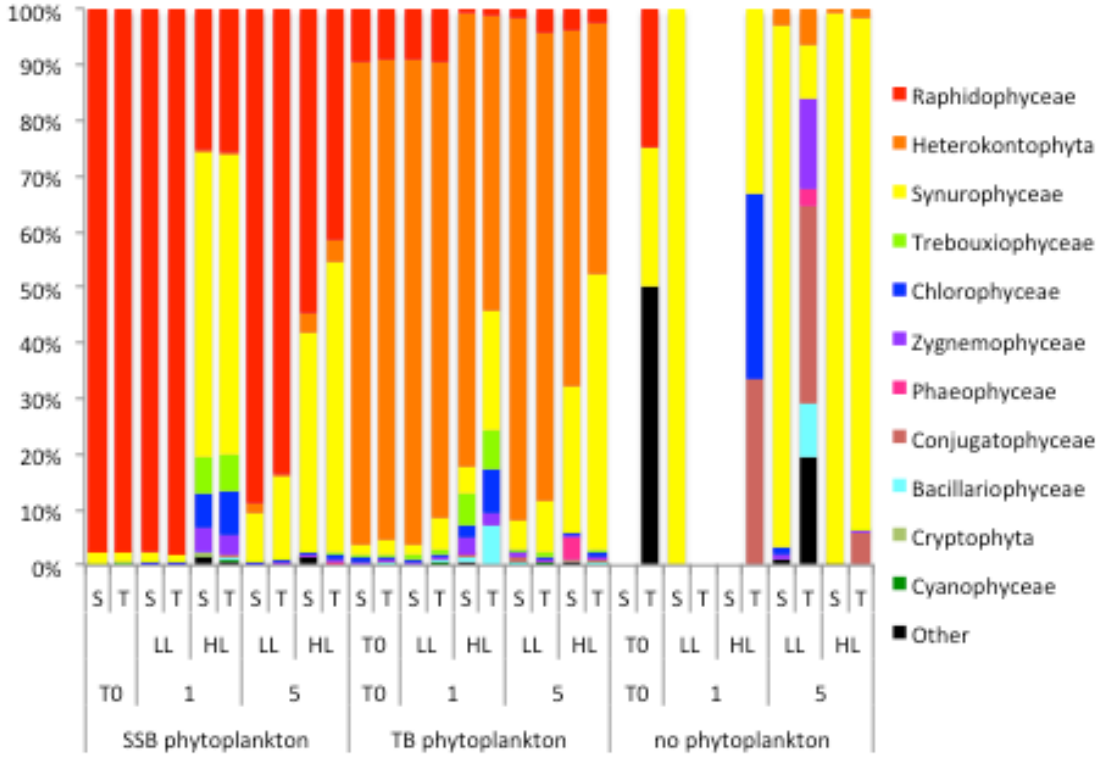

Figure C.4. Average percent of phytoplankton 23S rRNA plastid sequences belonging to different classes of phytoplankton in three replicate samples for each treatment. Samples are grouped by phytoplankton treatment, temperature (1=coldest temperature, 5=warmest temperature), light (LL=low light, HL=high light), and bacterial community (S=SSB, T=TB).

To determine how many 23S rRNA plastid pyrosequences were necessary to characterize phytoplankton composition in samples, sequences from samples from mesocosms incubated at the warmest temperature and high light were re-sampled down to 911, 750, 500, 250, and 100 sequences (Fig. B.4). Among-replicate differences were observed even when sampling as few as 100 sequences.

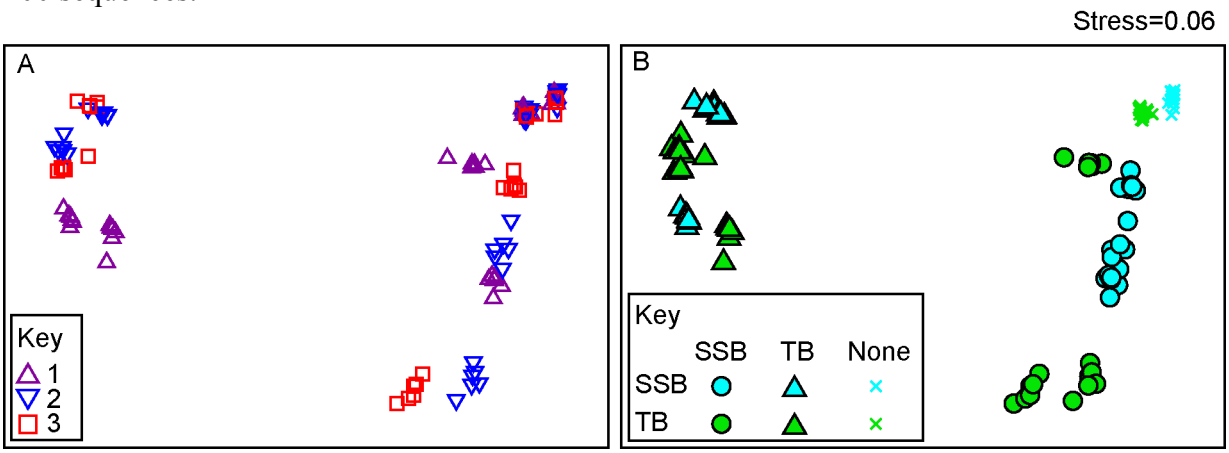

Figure C.5. Non-metric multidimensional scaling ordination of phytoplankton community composition following incubation at the warmest temperature and high light based on Bray-Curtis similarity of 23S rRNA plastid OTU relative abundances in resampled pyrosequencing operational taxonomic unit tables color coded by replicate (A) and bacteria and phytoplankton source (B).